Towards amperometric biosensors based on microbial ketone reductases for the measurement of novel substrates

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TOWARDS AMPEROMETRIC BIOSENSORS BASED ON MICROBIAL KETONE REDUCTASES FOR THE MEASUREMENT OF NOVEL SUBSTRATES

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ABSTRACT

This thesis considers the range of technological developments which are required in order to allow the creation of biosensors based upon keto reductases generated from microbial sources.

Using the oral anticoagulant warfarin as a model compound, a range of microorganisms were screened for an ability to reduce the aliphatic ketone group present in the drug to the corresponding alcohol. High performance liquid chromatography was used as the analysis method. Two successful microorganisms from the genus *Rhodococcus* were grown in a fermenter to produce 100g quantities of cells for further study.

The electrochemical problems which occur when creating a biosensor based on a reduction were considered. The electrochemical regeneration of enzymatically active reduced nicotinamide cofactors is difficult. An high performance liquid chromatography technique was developed to allow the determination of the products formed when the electrochemical reduction of nicotinamide adenine dinucleotide was carried out at different cathodic potentials at a mercury electrode.

In order to attempt to prevent the formation of nicotinamide adenine dinucleotide dimers and other inactive products during electrochemical reduction, a number of organometallic rhodium compounds were considered as possible electron transfer mediators and reaction modifiers. The synthesis of these compounds, based on the nitrogen containing heterocyclic ligands 2,2′-bipyridine and 2,2′;6′,2″-terpyridine and analogues, was attempted.

Methods for the incorporation of bacterial keto reductases, cofactors and mediators in to amperometric biosensors are discussed.

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This thesis is dedicated to Cathy

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1 INTRODUCTION, AIMS AND SCOPE

1.1 The Amperometric Biosensor

There has been intense research and interest in recent years in the emergent Biosensor technologies. A wide range of techniques are available to create devices which combine the specificity of biological systems with the high sensitivity of modern transducers. Several books[1,2,3] and many reviews of the subject have been published.[4,5,6,7,8]

A generic scheme for the construction of a biosensor is given in Figure 1.1

Many biosensors are based on the detection of products or reactants of an enzyme catalysed reaction. Device specificity is dependent upon the substrate specificity of the enzyme. Certain other sensors are based upon detecting the interaction between an antibody and its antigen. In theory, some of the available transduction methods should be able to detect almost any biological binding event.

Some possible transducer types/methodologies are given below:-

ELECTROCHEMICAL

Amperometric	Metal electrodes	} unmediated or mediated
	Carbon electrodes	
	Conducting organic salt electrodes	
Potentiometric	Ion selective electrodes (ISE)	
	Ion selective field effect transistors	
	Gas selective electrodes	

OPTICAL

Evanescent wave devices
Surface plasmon resonance

OTHER

Conductimetric
Impedimetric
Thermometric
Micrograviметric
Piezoelectric effect
Surface acoustic wave (SAW) devices

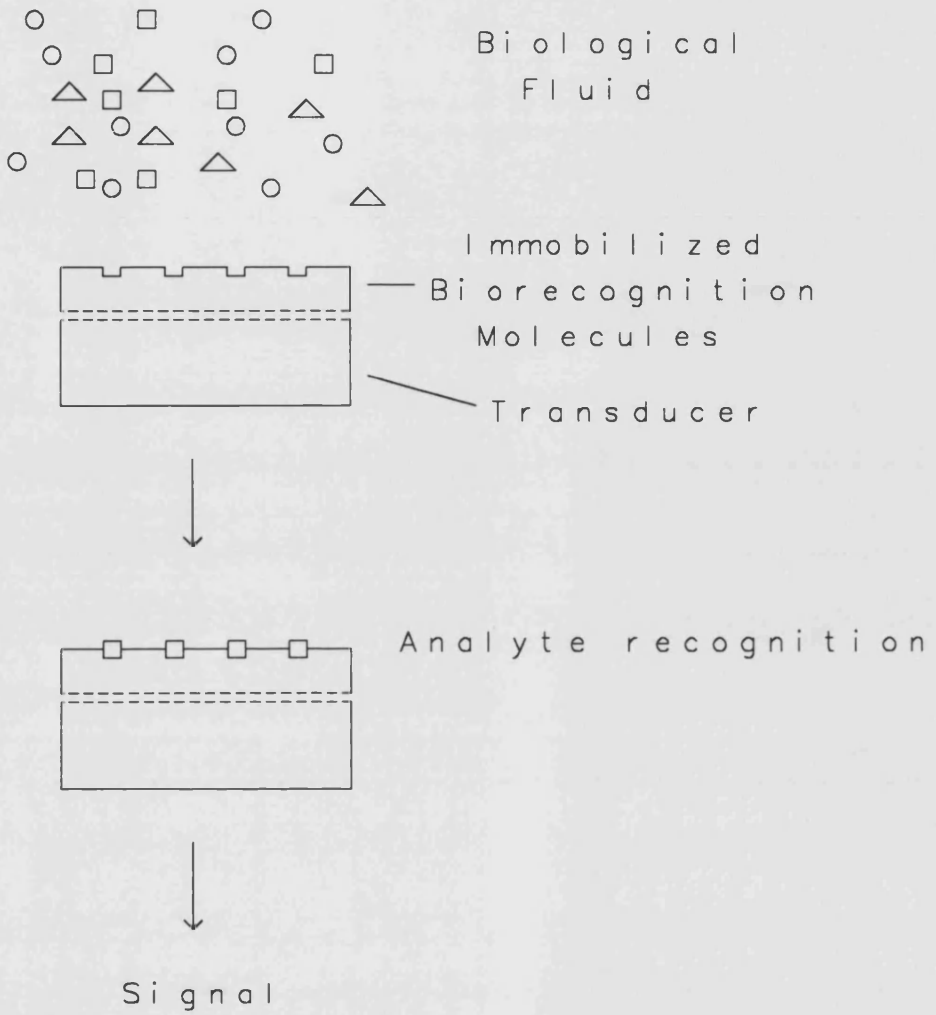


Figure 1.1 The generation of a signal in a generic biosensor

This introduction does not attempt to review all the different methods available for the creation of biosensors. It focuses its attention on the technological problems which have to be overcome in order to use enzymes for the creation of amperometric biosensors.

The basic requirements for an amperometric biosensor are:-

a) an enzyme which acts on its substrate to produce (or consume) a molecule which is capable of being reduced or oxidised (directly or indirectly) at a suitable electrode.

b) a method for immobilising the enzyme in close proximity to the electrode which retains the activity of the enzyme.

c) an electronic system capable of controlling the potential of the electrode and measuring the current produced by the oxidation or reduction.

Oxidation-reduction reactions are performed by enzymes which the International Union of Biochemistry classifies as oxidoreductases.

An oxidoreductase which has been studied in great detail for the construction of biosensors is Glucose Oxidase (GOD) due to its relatively low cost, high activity, good stability and due to the potentially lucrative applications of a simple glucose sensor. The development of the glucose sensor is explained in some detail as it serves to illustrate the types of technological hurdles which need to be overcome in order to develop other amperometric sensors.

The action that glucose oxidase has on glucose can be represented as follows:-

$$\text{GLUCOSE} + O_2 \xrightarrow{\text{GOD}} \text{GLUCONOLACTONE} + H_2O_2$$

The first glucose sensor[9] used an oxygen electrode (cathode) with GOD entrapped at its surface, to measure the local decrease in the oxygen tension at the electrode surface, this being proportional to the concentration of glucose in the solution. The problem with this arrangement was the dependence of the output current on the dissolved oxygen concentration of the glucose solution being analyzed. Below a certain dissolved oxygen

tension the current becomes proportional to the oxygen concentration rather than the glucose concentration.

An ingenious cylindrical sensor design[10] was developed and successfully used for the cathodic detection of 'oxygen decrease'. This electrode allowed oxygen to diffuse into the enzyme layer from two directions, but only allowed glucose to enter from one direction. This enabled glucose detection at lower concentrations of dissolved oxygen than in the original glucose sensors.

Most later sensors have relied upon the electrochemical detection of the hydrogen peroxide produced by oxidising it at an anode.[11]

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2e^-$$

Glucose oxidase contains two cofactor molecules of flavin adenine dinucleotide (FAD). These do not readily dissociate from the enzyme and therefore they are best described as prosthetic groups. Each group is capable of being reversibly reduced to give $FADH_2$.

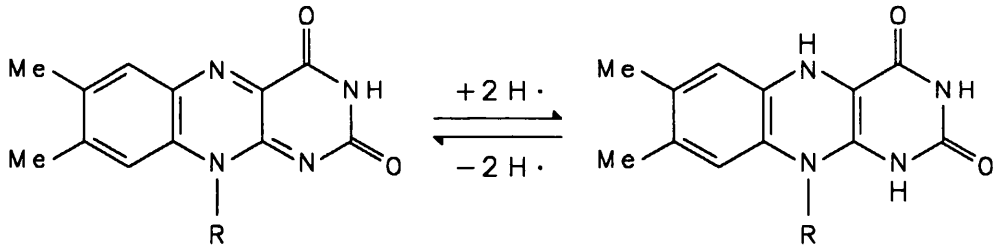


Figure 1.2 Oxidised and reduced forms of the isoalloxazine moiety of flavin adenine dinucleotide

Therefore, the conversion of glucose to gluconolactone by GOD can be more fully represented[12] by:-

$$\text{GOD-2FAD} + \text{glucose} \rightleftharpoons \text{GOD-2FADH}_2 + \text{gluconolactone}$$

$$\text{GOD-2FADH}_2 + \text{'electron acceptor'}_{\text{(oxidised)}} \rightleftharpoons \text{GOD-2FAD} + \text{'electron acceptor'}_{\text{(reduced)}}$$

As seen earlier, the 'natural' electron acceptor in solution is dissolved oxygen.

If glucose oxidase is immobilised at the surface of a suitable electrode which is poised at +0.7V (with respect to a Ag/AgCl electrode) a glucose sensor can be formed. The current flowing in a three electrode electrochemical cell, which has a glucose oxidase electrode as the working electrode, is proportional to the concentration of glucose in the cell solution. The reaction scheme for such a sensor is illustrated more clearly in Figure 1.3

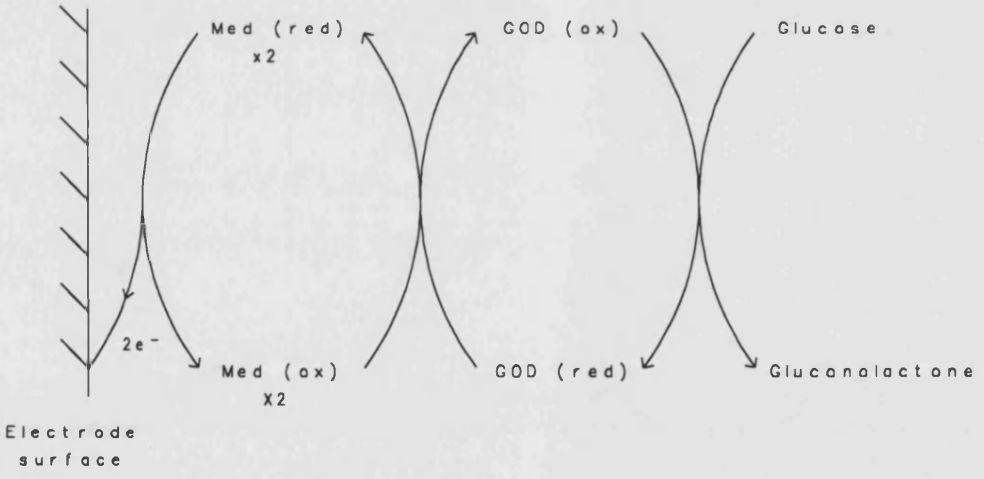


Figure 1.3 The reaction scheme for an amperometric glucose oxidase electrode.

GOD (red) and GOD (ox) are the reduced and oxidised forms of Glucose Oxidase. Med (red) and Med (ox) are the reduced and oxidised forms of the electron transfer mediator.

However such a scheme has several problems:-

a) if the solution has a very low oxygen concentration, the reaction rate will be controlled by the oxygen level rather than the glucose concentration.

b) the electrode must be held at approximately +0.7V[13]. This potential is in the region where the current produced from anodic decomposition of H_2O_2 is proportional to its concentration, but is below the potential at which solvent breakdown occurs (details of experimentally determined potentials for the electrolysis of water at a range of different electrode materials can be found in the book by Adams[14]).

At such a high potential, other biological molecules are capable of reacting at the electrode surface, the most notable of these being ascorbic acid.

In order to overcome these problems, the use of small redox molecules as electron transfer mediators has been attempted by a number of workers. Ferrocene derivatives have received the most research attention for this purpose (see section 1.3).

1.2 Enzyme Immobilisation Methodology and Electrode Types

An area which is crucial to the production of a functional glucose sensor is the method used to immobilise the enzyme (and any electron mediators) at the electrode surface. Many different techniques have been proposed and a small selection of these will be outlined below.

a) The earliest examples of immobilisation involved the use of simple semi-permeable membranes (eg dialysis membrane) to enclose the enzyme. In the first glucose sensor[9]

a membrane was also used between the platinum electrode surface and the enzyme to protect the platinum from contamination by the enzyme.

The currents produced by such designs are limited by the diffusion rates of substrates through these membranes. This can be a useful phenomenon as diffusion through such membranes is a concentration dependent process. Provided a sufficiently high concentration of the enzyme is present in the entrapped layer, a linearisation of the sensor response can be achieved[15]. If the membrane is thick however, it can take considerable time for a steady state to be achieved, leading to long response times. Also, detrimental changes to the local environment of the enzyme may occur due to the build up of products of the reaction within the layer enclosed by the membrane.

Some of the more recent designs of implantable sensor use membranes to entrap the enzyme layer[16] for bio-compatibility reasons. Very few of the electron transfer mediators so far considered for use in biosensors (see section 1.3) have been assessed for their toxicity in mammals[17,18].

b) Inert proteins such as albumin, have been cross-linked with glutaraldehyde, at the electrode surface, to form a matrix to entrap the enzyme. Synthetic gels, such as acrylamide / methacrylamide co-polymers have also been used[19].

c) Chemical bonding of the enzyme to various electrodes has been attempted. The method used by Ianniello and Yacynych[From Ref. 20] is shown in Figure 1.4

This method was used with both GOD and L-amino acid oxidase and produced electrodes which remained active for up to 30 days.

d) In an attempt to achieve direct electron transfer between the FAD moieties of flavoenzymes (such as Glucose oxidase) and an electrode, certain 'conducting organic salts' have been used as electrode materials (Kulys[21] and Kulys *et al*[22]).

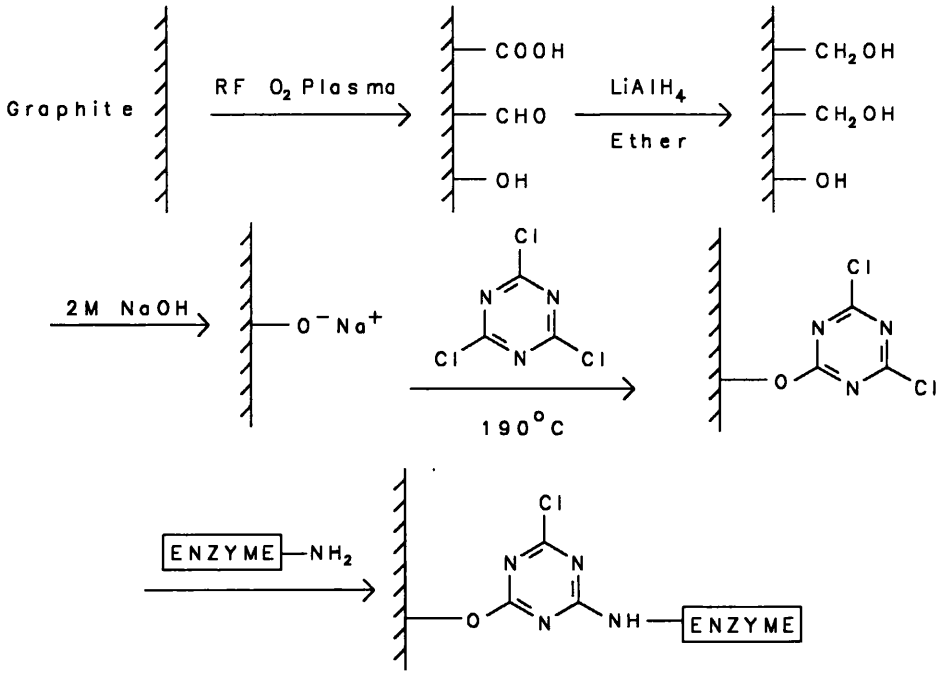


Figure 1.4 Enzyme immobilisation using chemical modification of graphite surface.

Albery *et al*[23,24] have used tetrathiafulvalinium tetracyanoquinodimethanide (TTF⁺TCNQ - Figure 1.5) to construct sensors with five different flavoenzymes; xanthine oxidase, D-amino acid oxidase, L-amino acid oxidase, choline oxidase and glucose oxidase[25].

Electrodes were constructed by forming a slurry of the salt and PVC in tetrahydrofuran. This was then used to fill a platinum cavity or used to coat a glassy carbon electrode. The enzyme (in an electrolyte solution) was then immobilised using a dialysis membrane secured with an O ring.

These sensors have the advantage of requiring no mediator at all (a mediator may be generated at the electrode surface by local dissolution of the electrode), and the

reduced forms of the enzymes can be oxidised directly at the electrode surface at potentials as low as +50mV.

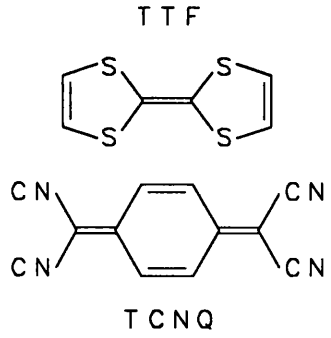


Figure 1.5 Structure of Tetrathiafulvaline (TTF) and Tetracyanoquinodimethane (TCNQ)

e) Recently, the use of polypyrrole as an enzyme entrapment matrix has generated a lot of interest.

It was found by Diaz and Castillo[26] in 1980 that pyrrole, dissolved in a variety of solvents, would readily polymerise at the surface of an electrode poised at between +0.8V and +1.3V. Further papers have discussed the variation in the mechanical, chemical, and electrical properties of the films produced.[27,28,29]

The use of polypyrrole in biosensors began in 1986, and if an enzyme is present when the electrochemical polymerisation of pyrrole takes place, some of the enzyme will be trapped at the electrode surface.[12,13,30]

The film produced is electrically conducting in its oxidised form and has an overall positive charge (approximately one positive charge for each four monomer rings). It consists mainly of linear chains which are joined by the α carbon atoms (Figure 1.6), although some cross-linking via the β carbons probably occurs.

The main advantages of polypyrrole film entrapment are:-

a) The immobilisation is a one step process.

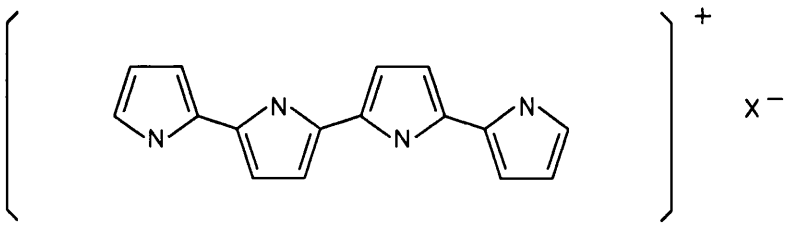


Figure 1.6 Diagrammatic representation of the conducting form of polypyrrole.

b) The film thickness is proportional to the amount of current passed, and can easily be controlled.

c) Electron mediators can be trapped at the same time as the enzyme.

d) The process should lend itself well to the production of very small sensors and to the production of multi-sensor probes.

e) It may be possible to construct multi-layer systems, with different enzymes in each layer, to produce coupled reactions. This may increase the range of substrates which can be measured.

f) It has been shown recently that direct electrochemical transfer is possible between the active centre of enzymes and conducting polypyrrole microtubules without the need for additional electron mediators[31].

g) Polypyrrole can be used to improve the performance of conducting organic salt electrodes[32].

Other molecules can be electrochemically polymerised and have also been used for enzyme immobilisation including N-methyl-pyrrole, aniline, phenol[6] and 1,3-diaminobenzene[33].

In addition to its use as an enzyme immobiliser, polypyrrole has also been studied for use in conductimetric sensors. A change in the local environment of the pyrrole film due to an enzyme reaction (eg. change in pH) can lead to a change in film conductivity which can be measured[34].

g) A novel and interesting technique involves the incorporation of the enzyme directly in to the electrode surface. By having the enzyme present during the electrochemical platinisation of a platinum metal electrode, Ikariyama *et al*[35] were able to create biosensors for glucose.
This technique lends itself well to the construction of miniature sensors using semiconductor fabrication techniques[36].

1.3 Electron Mediators

In order to function effectively, any electron mediator must have the following properties:-

a) Be stable in both reduced and oxidised forms.

b) Have a redox potential which shows little or no change in conditions of varying pH.

c) Be readily immobilised at the electrode surface.

d) Be able to operate at a potential which is as low as possible, in order that potentially interfering molecules cannot oxidise at the enzyme electrode.

e) React readily with the $FADH_2$ moiety of flavoenzymes.

Molecules which have been used extensively for this purpose are the Ferrocene derivatives. The structure of ferrocene is shown in Figure 1.7

One of the earliest reports of the use of ferrocene derivatives in amperometric biosensors came from Cass *et al*[37] in 1984. Since that time, many workers have studied the use of such electron mediation with a variety of degrees of success. In 1987 Matthews *et al*[38] published a paper describing a pen-sized amperometric blood glucose meter using ferrocene as a mediator.

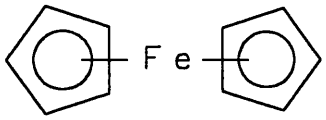


Figure 1.7 The structure of Ferrocene

1.3.1 Immobilisation of Ferrocene

Cass *et al*[37] deposited 1,1′-dimethylferrocene onto a graphite foil electrode by the evaporation of a solution (in toluene) at the foil surface. Glucose oxidase was then attached to the foil surface using carbodiimide covalent linkage and covered with a polycarbonate membrane. This method relies on the very low aqueous solubility of the ferrocene to keep it at the electrode surface. Electrodes prepared by this method have probably been the most successful ferrocene mediated glucose sensors to date. The electrode is operated at +160 mV (vs. saturated calomel electrode) which is 60 mV positive of E_0 for 1,1′-dimethyl-ferrocene. Low variation in output current is seen with variation of the oxygen tension of the analyte solution (approximately 4% change in output between a glucose solution purged

of oxygen and the same solution saturated with air). Addition of ascorbic acid at the normal level found in blood produced a current increase of around 4%, but a range of other possible interferents produced no observable change in current.

The ferrocene mediated glucose sensor described by Cass *et al* has been developed further[39], using a much smaller piece of graphite foil and a final electrode coating of polyurethane, to produce a potentially implantable sensor. A two electrode system was used employing a Ag/AgCl wire as a pseudo-reference electrode. This showed a higher dependence on dissolved oxygen concentration, and in addition the sensor gave lower output currents when operated in plasma than in buffer solution containing an equivalent glucose concentration.

A second approach to the problem of how to contain the ferrocene at the electrode surface is to bind it covalently to the glucose oxidase[40,41] (Figure 1.8 - from reference 40).

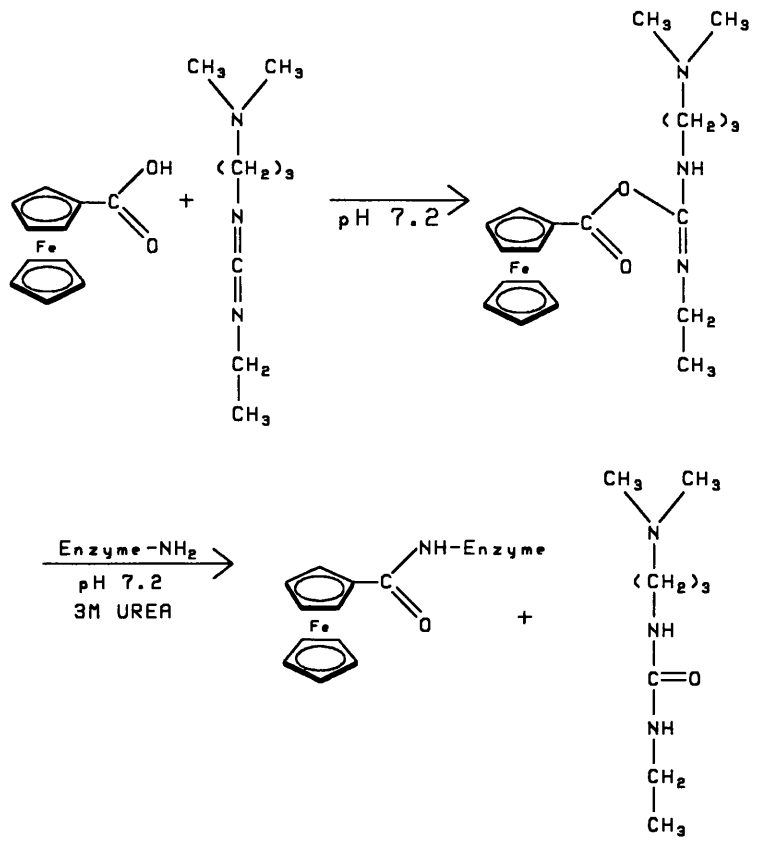


Figure 1.8 Steps involved in attaching electron relays to the protein backbone of an enzyme.

Atomic absorption spectroscopy showed that unmodified GOD contains two iron atoms per molecule, but after the covalent attachment of ferrocenecarboxylic acid the enzyme contained fourteen iron atoms per molecule[40]. The modification of glucose oxidase by ferroceneacetic acid and by ferrocenebutanoic acid has also been considered[41].

Chemical attachment of the mediator may be advantageous in the prevention of leaching of potentially toxic components from the biosensor[42].

The final method which has been considered for immobilisation of ferrocenes is the incorporation of the ferrocene into the matrix of an electrochemically prepared polymer which is being used to entrap the glucose oxidase[13,43].

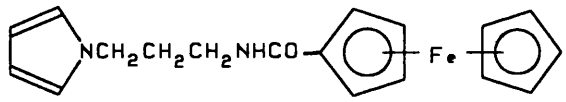
Electron mediators could conceivably be incorporated into polypyrrole films in several different ways:-

a) Attachment of mediator to a pre-formed film.

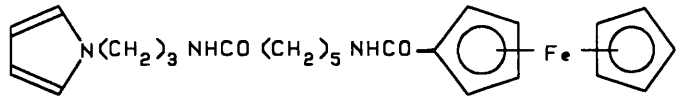
b) Pyrrole will incorporate anionic electron mediators during electro-polymerisation.

c) Attach mediator to the pyrrole monomer covalently.

Option (c) has been attempted by Foulds and Lowe[13,43] by synthesizing the ferrocene derivatives [(ferrocenyl)amidopropyl]pyrrole (FAPP) and ([(ferrocenyl)amido-pentyl]amidopropyl)pyrrole (FAPAPP) (Figure 1.9).



FAPP



FAPAPP

Figure 1.9 FAPP and FAPAPP

Any attachment of side chains to pyrrole at the α position resulted in a molecule which would not polymerise. Side chains at the β position would probably stop any cross-linkage

within the polymer and affect its mechanical strength. Therefore, the compounds were synthesised by N substitution of pyrrole.

When used to form homopolymers, these compounds gave very poor, thin films if prepared using constant potential deposition but more satisfactory films were produced by cycling the potential between 0V and +1.0V at $100mVs^{-1}$. However, sensors formed in this way were not independent of the dissolved oxygen concentration, and within two days of fabrication they showed no response in the absence of oxygen. In addition to this, a potential of +300mV was required to re-oxidise the bound ferrocene. Co-polymers of FAPP and FAPAPP with pyrrole were also considered.

1.3.2 Theoretical Aspects of Electron Mediation

In order to allow a mediated glucose sensor to operate at a potential which is sufficiently low, so that the oxidation currents produced by any interfering compounds are insignificant, the redox potential of the mediator (as indicated by the standard electrode potential [E_o] or by the half wave potential [$E_{½}$] in cyclic voltammetry) should be as low as possible. Reported values from the literature[13,37,44] are given in the table below (in order).

E_o and $E_{½}$ values for ferrocene derivatives from the literature.	
ferrocene derivative	**$E_{½}$/mV *vs.* Ag/AgCl**
FAPP	165
FAPAPP	170
poly FAPP	210
poly FAPAPP	250
	E_o/mV *vs.* SCE
1,1′-dimethyl	100
ferrocene	165
vinyl	250
carboxy	275
1,1′-dicarboxy	285
(dimethylamino)methyl	400
	$E_{½}$/mV *vs.* SCE
1,1′-dicarboxylic	395
monocarboxylic	275
hydroxymethyl	185
2-aminoethyl	200

Even though the over potential required for a particular derivative to act as a mediator may be low, the rate at which the derivative is able to transfer electrons from the reduced GOD to the electrode may be poor.

A useful indicator of mediator efficiency is the second order rate constant for the reaction between GOD and the mediator (k_s).

The second order rate constant can be derived from cyclic voltammograms obtained under specific conditions, and by applying the mathematical theory for an overall reaction scheme which consists of an irreversible catalytic reaction following a reversible charge transfer (Nicholson and Shain[45];case VII).

As seen before, the reaction scheme occurring at the surface of a ferrocene mediated glucose sensor can be represented by:-

$$\text{GOD-2FAD} + \text{glucose} \longrightarrow \text{GOD-2FADH}_2 + \text{gluconolactone}$$

$$\text{GOD-2FADH}_2 + 2\text{FeCp}_2\text{R}^+ \longrightarrow \text{GOD-2FAD} + 2\text{FeCp}_2\text{R} + 2\text{H}^+$$

$$2\text{FeCp}_2\text{R} \rightleftharpoons 2\text{FeCp}_2\text{R}^+ + 2e^-$$

(FeCp_2R represents the ferrocene derivative)

If the reaction of the ferrocene at the electrode surface is fast compared with its reaction with GOD, and there is sufficient excess of glucose in solution (in order that the enzyme is maintained in a fully reduced state) then the reaction scheme can be reduced to the equations given below and the mathematical theory can be applied.

$$\text{R} - e^- \rightleftharpoons \text{O};\ \text{O} + \text{Z} \xrightarrow{k_f} \text{R}$$

where R and O are the reduced and oxidised forms of the ferrocene, Z is the reduced enzyme and k_f is the pseudo-first-order rate constant.

From experimentally determined parameters it is possible (using Figure 14 of reference 45) to calculate k_f for a range of glucose oxidase concentrations. k_s is then found from the gradient of a plot of k_f *vs.* GOD concentration.

The second order rate constants for some of the ferrocene derivatives are shown below.

k_s values for ferrocene derivatives from the literature	
ferrocene derivative	**$10^{-5}k_s$ /L mol s^{-1}**
FAPP	6.70
FAPAPP	21.00
1,1′-dimethyl	0.77
ferrocene	0.26
vinyl	0.30
carboxy	2.01
1,1′-dicarboxy	0.26
(dimethylamino)methyl	5.25
1,1′-dicarboxylic	0.26
monocarboxylic	1.80
hydroxymethyl	9.00
2-aminoethyl	44.00

The second order rate constant k_s for the reaction of reduced GOD with oxygen has been determined by Weibel and Bright[46].

$$k_s = 1.5 \times 10^6 \text{ l mol}^{-1} \text{ s}^{-1}$$

Several interesting points can be observed from this and the data in the table:-

a) There is no correlation between redox potential (E_0 or $E_{1/2}$) and the second order rate constant k_s for the various mediators.

b) For the majority of mediators, their reaction rate with GOD is lower than that of oxygen.

c) The highest value of k_s is that obtained for 2-aminoethyl ferrocene. This mediator is positively charged at pH 7 (pK_a=9.5) and suggests that the active site of glucose oxidase is negatively charged.

d) FAPAPP has a high rate constant (above that for oxygen) despite having a relatively bulky side chain attached to one of the cyclopentadiene rings of the ferrocene moiety.

1.4 Measuring Drug Levels Using Amperometric Biosensors

Amperometric biosensors are potentially ideal for the measurement of drug molecule levels in a variety of solutions. They have the major advantage over most other types of biosensor (excluding potentiometric systems) in that they can produce continuous readings.

Monitoring of clinically useful analytes in a variety of biological fluids by biosensors can be considered in a range of different situations listed below:-

a) Chronic conditions in which a routine test by the patient can allow adjustment of a drug dosage. The classic example of this is the measurement of blood and/or urine glucose levels by diabetics, enabling self adjustment of insulin dosage.

b) Chronic conditions where the ability to continuously monitor a drug level would allow control of a drug delivery system. This would create a feedback loop similar in principle to many of the regulatory mechanisms found in the body. Such a system would be extremely useful in treatments which require the use of drugs which have a low therapeutic index or in conditions where poor drug level control over prolonged periods can create additional problems. It may also be of use in monitoring of hormone replacement therapy, especially when natural levels of the hormone would normally vary depending upon the time of day or time of month. This sort of variation could be pre-programmed into such a system.

c) Acute emergency situations where it is necessary to know the blood levels of a drug extremely quickly. eg. poisoning or overdosage.

d) Measurement of drug levels during operations and in intensive care situations, allowing feedback control of blood levels by direct regulation of drug infusion rates.

e) Single measurements, either by the patient or within a doctors surgery, for diagnostic purposes. A comparison can be made here to existing products such as pregnancy testing kits.

Many newer types of non-amperometric sensors are based on measuring a change in a physical property associated with the formation of an antigen-antibody complex. Whilst these methods have the ability to measure concentrations of substances at several orders of magnitude lower than current amperometric systems, they are generally single use methods. Once an antigen has bound to an antibody it is difficult to remove it. Dissociation of the complex is slow, so limiting the possibility of using such a device for dynamic measurements.

It is likely that some of the non-amperometric biosensors will be extremely useful for measurements in categories a), c) and e) above.

The best prospects for creating amperometric biosensors which are clinically useful are in areas which require continuous analyte monitoring.

Few enzymes exist which specifically act upon a drug molecule, and even fewer which are also redox enzymes. However, in the future, with advances in protein engineering it may be possible to tailor make specific enzymes[47], or to increase the specificity of certain enzymes or synthetic chemical catalysts by linking them to antibodies[48].

1.4.1 Current Drug Biosensor Designs

Although progress is being made towards reliable *in vivo* measurements of clinically useful endogenous analytes[49], fewer articles exist in the literature which demonstrate useful biosensors for the measurement of exogenous drug molecules. A recent review by Guilbault and Schmidt[50] showed that much of the current research interest is being directed towards drugs of abuse.

One area which shows considerable promise is to link immunoassay to an amperometric detection system. Such a system has been developed for lidocaine by di Gleria *et al.*[51] and a schematic diagram of this is shown in Figure 1.10

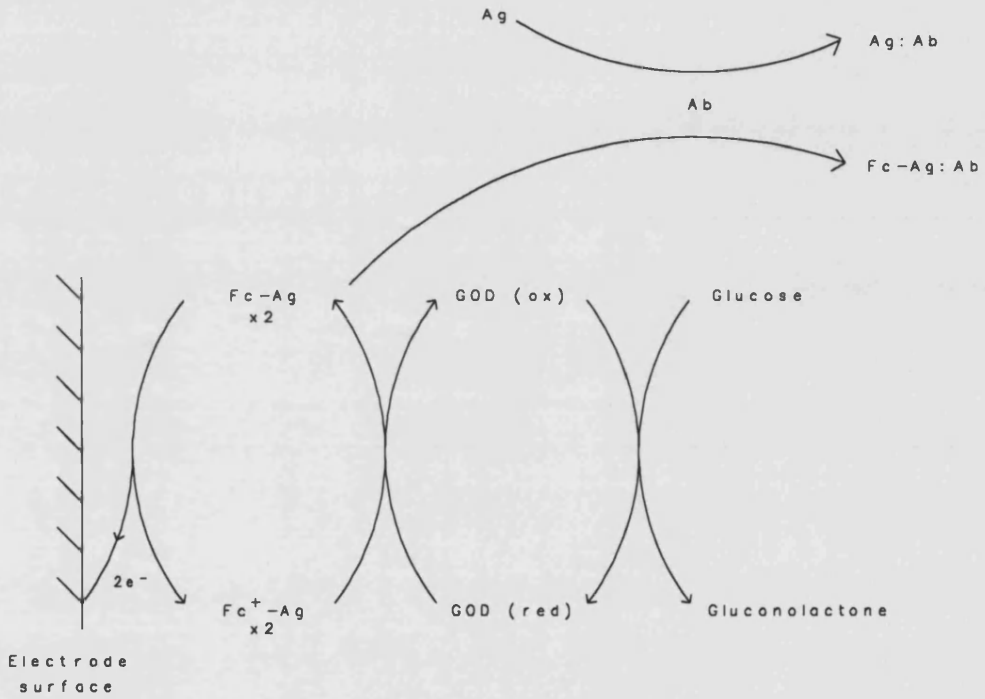


Figure 1.10 Schematic diagram of a homogenous amperometric immunoassay.
Ag refers to the antigen (in this case lidocaine);Fc-Ag is the ferrocene-drug conjugate;
Ab is antibody to lidocaine)

The assay relies upon the synthesis of a ferrocene-drug conjugate which retains the electron mediation properties of ferrocene. Glucose and a known quantity of antibody to the drug have to be added to a known volume of the solution being analyzed.

When the antibody binds to the ferrocene-drug conjugate, electron mediation is inhibited. Free drug in the analyte solution competes for the antibody, releasing free ferrocene-drug conjugate and increasing electron mediation.

In theory, this sort of approach could be used for any small molecule to which an antibody can be raised, and which can be effectively attached to an electron mediator whilst retaining the mediator activity. However, this type of reaction scheme is unfortunately subject to the same limitations as for the non-amperometric immunoassays. The technique is further limited by the necessity for accurate volume measurements and by the need to add further reagents to the analyte solution. These last two limitations can be partially overcome by the use of clever packaging of the biosensor (as demonstrated in capillary fill devices[52]), but the inability of such a device to perform continuous measurements still remains.

1.5 Aims and Scope

A summary of amperometric biosensor development has been given in this introduction in order to familiarise the reader with the concepts involved. Many groups of researchers are working on improvements to immobilisation technology, electron mediators and electrode materials. Ideally, electrode materials will be developed which allow the concept of a mediator to become redundant one.

However, a whole sub-class of enzymes exist which have so far been poorly exploited for biosensor creation. This is because these oxidoreductases perform reductions which lead to oxidised nicotinamide adenine dinucleotide cofactors which cannot easily be regenerated at an electrode surface. The mechanisms behind this are given more fully in the introduction to chapter 5.

Research is being performed in other scientific areas which can provide useful information and materials for biosensor production. One of these areas is that of microbial biotransformations. When these biotransformations involve reductions or oxidations the microbe involved is worthy of study for the creation of biosensors.

The direct aims of this research were therefore:-

1) to develop the necessary instrumentation and in-house electrode fabrication technologies required for the production of biosensors.

2) to review the literature and to screen microorganisms for their abilities to reduce or oxidise drug molecules with a view to using enzyme extracts for the creation of drug biosensors.

3) to look at ways in which it might be possible to regenerate enzymatically active reduced nicotinamide co-factors by electrochemical means, leading to an expansion in the range of enzymes available for the creation of biosensors.

2 MATERIALS, STANDARD METHODS AND INSTRUMENTATION

2.1 General materials

2.1.1 Water

In all electrochemical, HPLC (high performance liquid chromatography) and synthetic chemistry experiments, the water used to prepare solutions had been purified using a Milli RO (Reverse osmosis) system followed by a Milli Super Q (ion exchange) system. This water is referred to in the text as "RO water". Laboratory distilled water was used in all other situations e.g. preparing bacterial growth media.

2.1.2 HPLC solutions

All aqueous fractions of HPLC mobile phase solutions were filtered through a 0.45μm pore size cellulose acetate membrane filter (Whatman Ltd, Maidstone, Kent) prior to mixing with any other HPLC grade solvent. Mobile phase solutions were sparged with argon or oxygen free nitrogen (through a metal HPLC solvent filter) for at least 10 minutes before use. Compressed gas cylinders were obtained from B.O.C. Ltd, Herts, England.

2.2 Standard Methods

2.2.1 Measurement of pH

pH measurements were taken using either a Radiometer PHM 64 research pH meter (Radiometer Ltd., Crawley, West Sussex) or a Philips 9410 pH meter (Philips Ltd. Cambridge). A new pH electrode was obtained (Russell CWL/NS14/15/85) which was designed to fit the Metrohm electrochemical cell (see later).

Calibration was performed using the most appropriate two solutions from the three NBS primary standards given below[53]:-

1) Potassium hydrogen phthalate 0.05m in RO water (pH 4.002 at 20°C, pH 4.008 at 25°C)

2) Potassium dihydrogen phosphate 0.025m with disodium hydrogen phosphate 0.025m in RO water (pH 6.881 at 20°C, pH 6.865 at 25°C)

3) Borax 0.01m in RO water (pH 9.225 at 20°C, pH 9.180 at 25°C).

Buffer salts of A.C.S. reagent grade were obtained from the Aldridge Chemical Co. Ltd. and were oven dried and stored in a desiccator as appropriate. Most of the pH measurements in this thesis were not critical (molar solutions were used instead of molal solutions) and the meters were calibrated to a second decimal place only.

2.2.2 Solution volume measurement

Volumes were measured using class B glass pipettes or by Gilson automatic pipettes as appropriate. 5ml, 1ml and 200µl automatic pipettes with disposable nylon tips were used. These were periodically calibrated by weighing the volume of water given out by the pipette at 20%, 40%, 60%, 80% and 100% of maximum volume using a standardised pipetting technique. Five replicates were performed at each point.

Rather than try to adjust the pipettes to give the correct reading, a linear regression was performed. The resulting equation was used to work out the correct setting required on the pipette to give the desired volume for any critical measurements.

2.3 Electrochemical Instrumentation

Electrochemical measurements were taken using :-

Metrohm 626 Polarecord (polarograph) with Metrohm 612 VA Scanner.

(Metrohm Ltd., Herisau, Switzerland).

The Polarecord was used in its DC mode connected to a three electrode electrochemical cell. In this mode it behaves as a potentiostat, with the voltage at the working electrode being controlled by external input from the VA scanner.

The VA scanner could be used to apply a fixed voltage or it could be used to perform linear or cyclic sweep voltammetry (Figure 2.1).

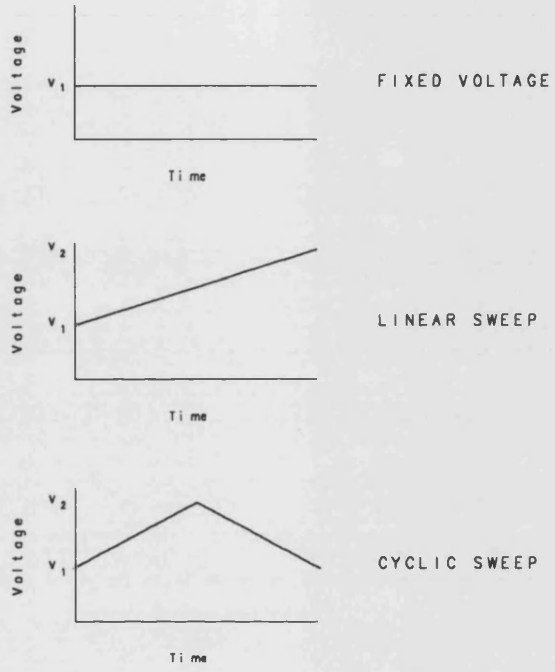


Figure 2.1 Voltage / Time profiles produced by the Metrohm VA Scanner.

For the purpose of recording experimental results, the Polarecord gives a voltage output between -1v and +1v proportional to the current flowing at the working electrode. This voltage, and the voltage of the working electrode were measured by using a 2 channel 12 bit analogue to digital converter interfaced to an Acorn Archimedes A310 microcomputer. In addition to this, the microcomputer was used to control scanner function.

The analogue to digital converter was designed and built by Sean Hagan (postgraduate student, School of Electrical Engineering, The University of Bath). Figure 2.2 is a schematic diagram of the equipment interconnections.

A fast X/Y recorder could have been used for recording of voltammograms up to scan rates of approximately 300 mVs^{-1}, and a storage oscilloscope used for higher scan rates. However, computerisation of the system has the advantage of allowing easier data handling and the benefit of more precise marking of axis details.

The equipment described above had a selectable current sensitivity between ±25 milliamps full scale deflection (f.s.d.) and ±6.25 nanoamps f.s.d. Careful screening of cables and shielding of the cell ensured that induced currents from external sources such as mains supplies were not a problem, even at high current sensitivities. However, analysis of the output voltage of the polarecord using an oscilloscope showed the presence of small spikes due to un-smoothed operational amplifier switching potentials within the unit. At most current sensitivities, and at slow scan rates these did not cause any significant problems.

The computer program for the Acorn Archimedes A310 microcomputer, to control and receive data from the Metrohm 626 polarograph and 612 VA scanner, is given in appendix 1.

The program is written in Acorn BASIC 5 and uses a short machine code routine to access the A to D converter. The mnemonic form of the routine (prior to assembly) is contained in a procedure called code, starting at line 6160.

The routine is called during a procedure called Read2 at line 3900. The routine takes a set number of samples from each channel and returns an additive value for each channel into variables X% and Y% at line 4020.

The number of samples taken during each call to the routine is controlled by the value contained in the variable VAR%, calculated at line 3930. The values contained in X% and

Y% are then divided by VAR% to give an average. This averaging provides a filtering effect against background noise, but the degree of this 'software filtering' depends upon VAR%.

The value of VAR% depends on the initial and final voltage for a sweep, and upon the sweep rate. At high sweep rates the value of VAR% is small in order that sufficient data points are collected to be able to plot a meaningful voltammogram.

Data from the program is written to floppy disk in a format which is recognised by a computer aided design (CAD) program (Design CAD, American Small Business Computers Inc.). This allows output of the voltammogram on a variety of printers or plotters at an accurate, specified scale. Output to disk in encapsulated postscript format was also included to allow direct incorporation of images into most currently available word processors.

The computer program was modified to allow it to capture data from high performance liquid chromatography experiments. The program listing for this is also given in appendix one.

2.4 Thin Film Electrode Development

In order for amperometric biosensors to become useful tools for monitoring clinical analytes in acute or chronic medical conditions, several advances in design need to be realised.

To enable devices to be made sufficiently small for such use, it is necessary for :-

1) optimisation of the interface between the biological recognition system and the electrode (discussed in the introduction).

2) development of miniaturised potentiostatic circuitry capable of measuring very low currents, and possibly relaying signals to a remote site for analysis.

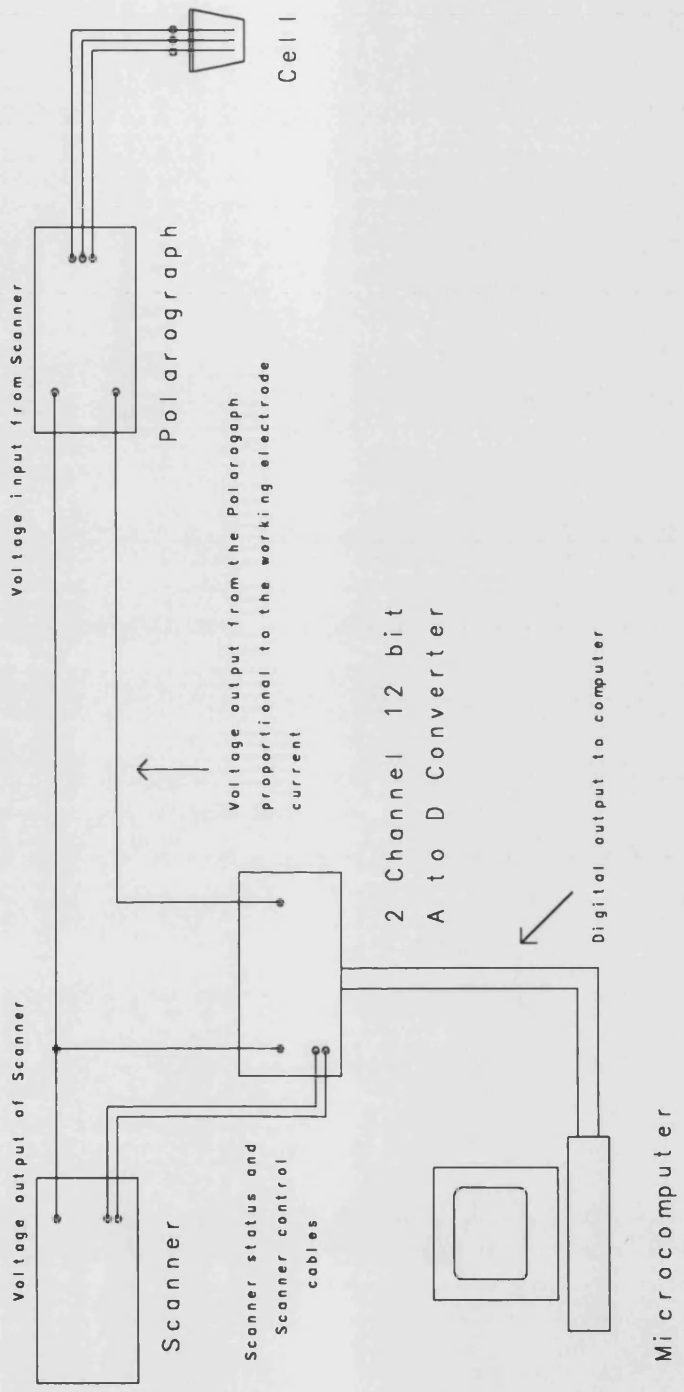


Figure 2.2 Block diagram showing the electrochemical instrumentation.

3) electrode design compatible with the electronic components. If very low current is to be measured, it is important that the electrical connections to the electrodes do not pick up any induced currents. To achieve this, they should be as short as possible, or ideally not exist as all. This concept can be accomplished by placing the electrode on the same silicon wafer as the integrated circuitry for the potentiostat and has been addressed by several groups[54,55,56].

For development work, it is necessary for electrodes to be macroscopic, but it was decided to investigate the use of integrated circuit fabrication technology to make them.

Electrode materials such as graphite or glassy carbon are not compatible with this sort of technology, however the coating of metals onto silicon can be done using a variety of well established techniques.[57]

Several recent reports of electrode construction using planar IC fabrication methods have been published.[58,59,60,61,62] Although the metallisation procedures are well documented there is little information on the stability of such devices (and in particular, the stability of insulator materials) on exposure to aqueous systems. Many different materials have been used as insulators, particularly in the field of potentiometric devices based on the use of ion sensitive field effect transistors (ISFETs). Silicon dioxide[63] has been used, but the material most commonly used in more recent ISFET devices is silicon nitride (Si_3N_4).[64,65,66] This may be due to its superior ion-selective properties rather than for stability reasons.

In the manufacture of planar amperometric electrodes polyimide[58], Tantalum oxide (Ta_2O_5) [60], epoxy resin[61] and negative photoresist[62] have been used as insulators. With all of the above designs, with the exception of the design of Sansen *et al*[58], the electrode area is defined without surrounding it with an insulator. The metallic layers are simply patterned onto a substrate (silicon, sapphire or glass) using either a lift off technique or by using wet etching. Insulator is then placed over the areas of metallisation which can

be considered to form the 'wires' to the electrode. This sort of methodology has the disadvantage of producing more variable electrode areas, particularly when epoxy resin is used as an insulator because application of this material is a manual process. In addition, the edge of underlying metal layers, used to improve adhesion of the electrode metal, will be exposed to solution (admittedly tiny areas).

The design shown in Figure 2.3 is that of a platinum working electrode, the exposed platinum region having a geometric surface area of 3.6mm^2.

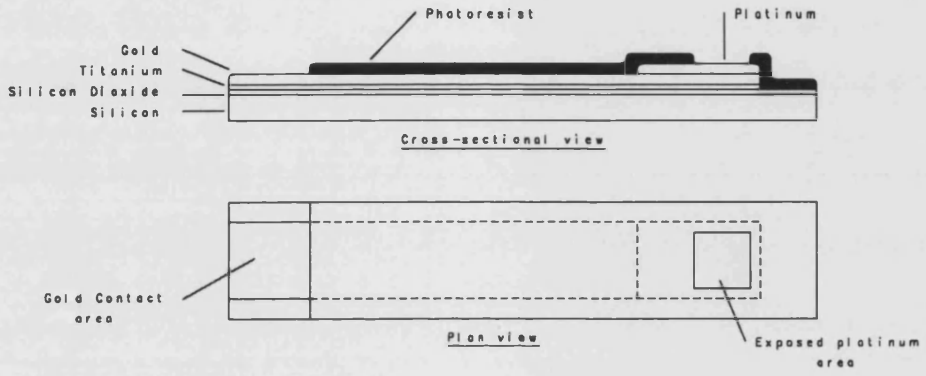


Figure 2.3 Cross-sectional and plan views of the working electrode (not to scale).

It was constructed by coating successive layers onto a piece of silicon which had been cut to size. Brief details are given below:-

Silicon wafer rejected by the electronics industry was made use of, the section to be used for the electrode being cut from a flawless area of the wafer. This had a layer of silicon dioxide already on its surface which had been produced under controlled conditions by the manufacturer of the wafer.

After cleaning of the surface, a positive photoresist was applied. This was then exposed to ultra-violet light after a mask, created using standard photolithographic techniques, was

positioned over the wafer. The photoresist exposed to the UV light was then washed away. This left exposed the area of silicon dioxide to be coated with the layers of titanium, gold and platinum.

Titanium was coated onto the surface using a thermal evaporation technique. This layer was thin (approximately 30nm) and was only present to improve the adhesion of subsequent layers.

The next layer consisted of a 150nm coating of gold, again applied using thermal evaporation. This was used to form a conductor from the contact end of the electrode to the platinum.

The final metallic layer is a 150nm coating of platinum, applied using ion beam coating. This technique was used because the thermal evaporation of platinum is difficult to achieve. However, ion beam coating is a slow process, and only small areas (approximately $1cm^2$ with the available equipment) could be coated. This was the reason for using the thermally evaporated gold layer as a conductor. On a much smaller electrode, the gold layer would be unnecessary.

The final process was to dissolve away (lift off) the positive photoresist, then to apply a negative photoresist (using another mask) to act as an insulator (Kodak microresist 752, Kodak Ltd, Hemel Hempstead). This photoresist layer covered the whole electrode apart from the required platinum area and the contact area.

All electrodes were fabricated by Trevor Ryan of the Department of Electrical Engineering, University of Bath.

Earlier designs of this electrode used a silicon dioxide layer as an insulator material. However, this soon broke down on exposure to dilute aqueous solutions of hydrogen peroxide in 0.1M potassium chloride. This could have been caused by poor adhesion of the silicon dioxide to metallic layers below, or it could possibly have been due to microcrack formation within the layer (Cohen *et al*[67]).

The fabrication technique outlined (and available equipment) only allowed the production of one electrode at a time. With smaller electrodes envisaged in the future, many hundreds or thousands of electrodes with their associated electronic circuitry, could be produced from one silicon wafer. These would then be cut out from the wafer and packaged to produce discrete biosensors.

2.4.1 Testing the electrodes

The electrodes produced were examined using two electrochemical experiments to determine whether their response was equivalent to that of platinum wire, and to test the stability of the photoresist insulator.

2.4.1.1 Materials

Potassium chloride (99+ % A.C.S reagent), potassium ferricyanide (99+ % A.C.S. Reagent) and 0.5mm diameter platinum wire 99.99% were obtained from the Aldrich Chemical Co. Ltd., Dorset, England.

2.4.1.2 Other Electrodes

Reference electrode: Radiometer K401 Saturated Calomel (SCE) (Radiometer UK Ltd, Crawley, West Sussex)

Counter Electrode: 1cm^2 Platinum Foil (one half of Metrohm EA 210 titration electrode)

2.4.1.3 Electrochemical cell

The orientation of the various electrodes within the electrochemical cell is shown in Figure 2.4

The working electrode holder consisted of a modified printed circuit board edge connector, in which the electrode was mounted, soldered onto a piece of Vero board. Epoxy resin was then used to protect the holder from splashes and to seal it into a plastic stopper.

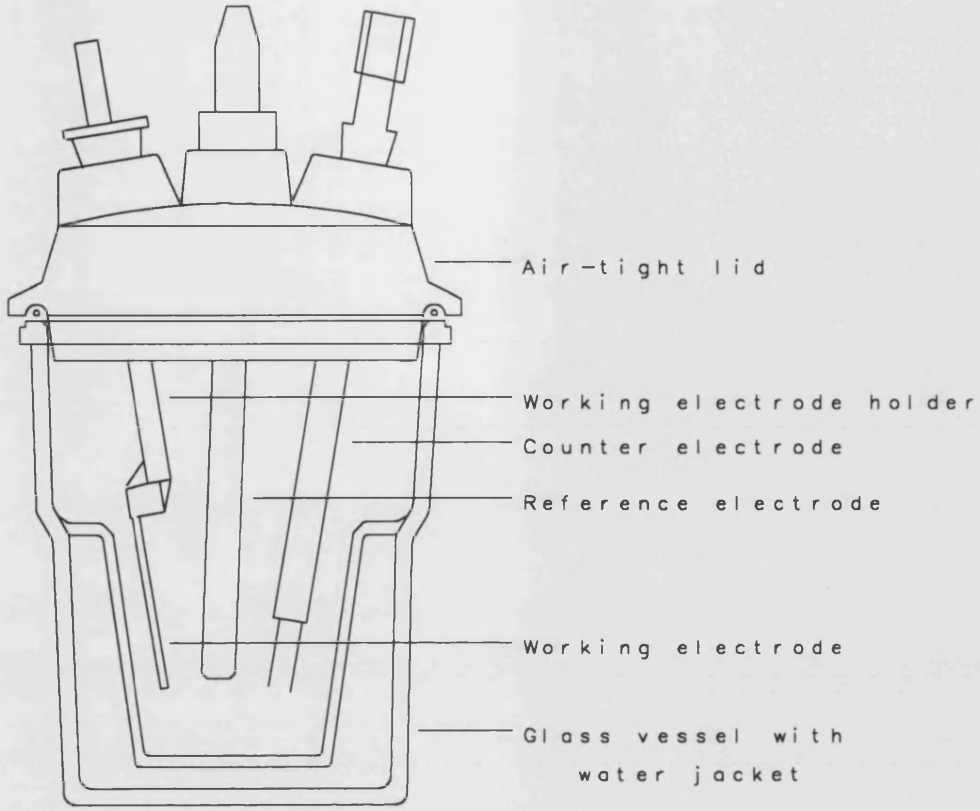


Figure 2.4 Electrochemical cell design.

The air-tight lid of the cell had a two additional ports not shown in Figure 2.4. One of these had a dip tube fitted to allow de-aeration of the solution by bubbling with pre-humidified nitrogen. The nitrogen was re-directed to flow over the solution during measurements.

The temperature of the water re-circulating through the water jacket of the cell was controlled by a Grant SU6 water bath heater, working against a Haake EK12 refrigeration unit.

2.4.1.4 Electrode response to potassium ferricyanide

The behaviour of platinum film electrodes was compared with the behaviour of a platinum wire electrode of a similar surface area by examining cyclic voltammograms of

potassium ferricyanide (potassium hexacyanoferrate III). A solution of this compound at a concentration of $10mM.l^{-1}$, with $1M.l^{-1}$ of potassium chloride (background electrolyte) in RO water was prepared. 20ml was added to the electrochemical cell and was de-oxygenated by bubbling with nitrogen for 15 minutes. Once temperature equilibration had occurred (25°C) the working electrode potential was held at +600mV, and the background current allowed to decay to a low value. A cyclic voltammogram was then recorded by taking the electrode potential down to -100mv then back to +600mv at $50mV.sec^{-1}$.

The cyclic voltammogram for the platinum film electrode is shown in Figure 2.5.

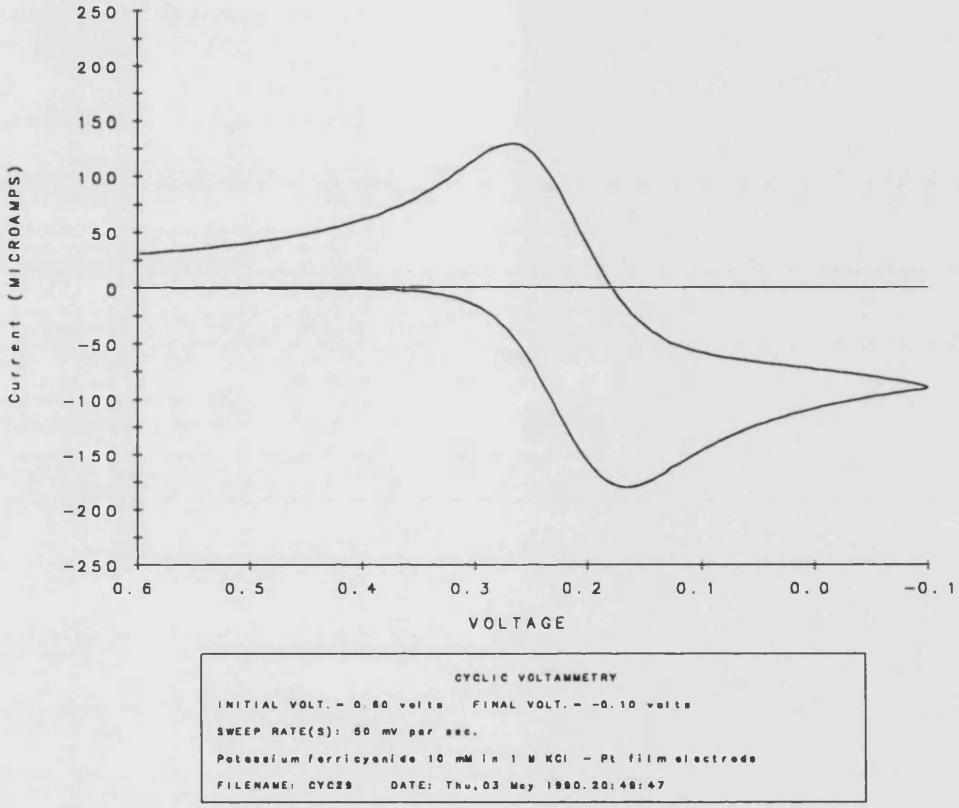


Figure 2.5 Cyclic voltammogram of potassium ferricyanide at a thin film platinum electrode

The platinum wire electrode was constructed by sealing a short piece of 0.5mm diameter wire into the end of a piece of glass tubing (5mm external diameter). Soda lime glass was used because it has a linear expansion coefficient similar to that of platinum. The seal

between the glass and the metal was examined microscopically for evidence of cracks. Once a good seal had been established the length of wire protruding from the glass was trimmed to 8mm leaving a geometric surface area of approximately 13mm^2 (platinum film surface area = 3.6 x 3.6 = 12.96mm^2). The cyclic voltammogram for the platinum wire electrode is given in Figure 2.6. As can be seen, the two voltammograms are virtually indistinguishable.

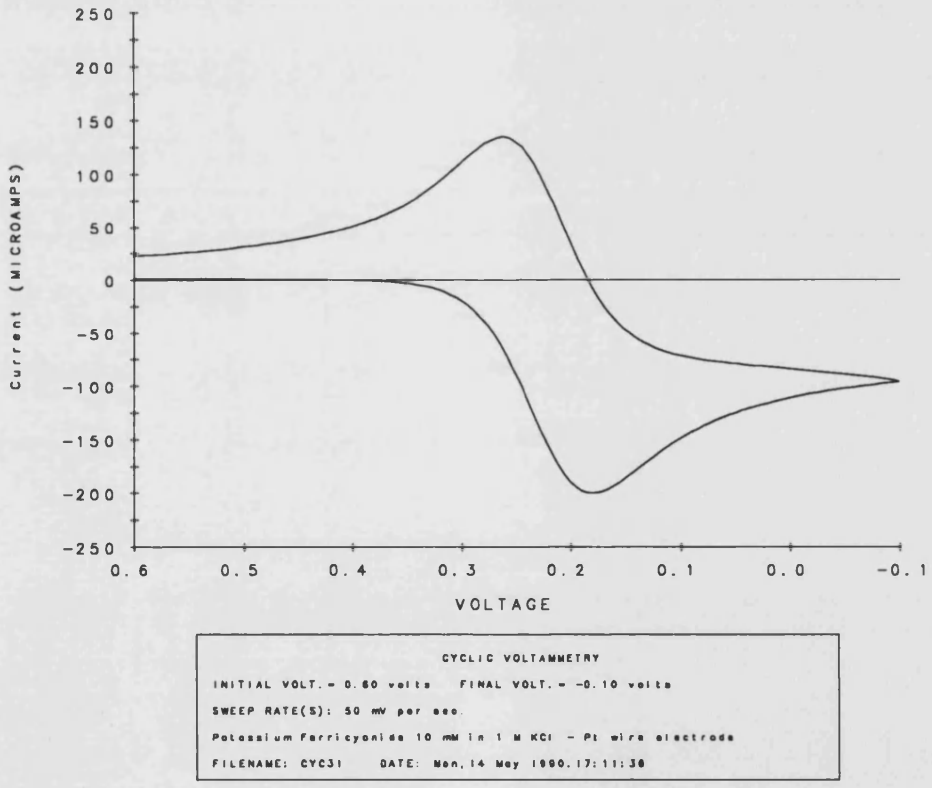


Figure 2.6 Cyclic voltammogram for potassium ferricyanide at a platinum wire electrode

2.4.1.5 Insulator stability

The stability of the photoresist used as an electrode insulator was assessed. The easiest way to do this was to monitor any change in the electrochemically measured surface area of the electrode with exposure to solution.

The peak cathodic current i_{pc} (as recorded using cyclic voltammetry) for the reversible reduction of O to R

$$O + ne^- \rightleftharpoons R$$

where only O is initially present in solution is given by:-

$$i_{pc} = (2.69 \times 10^5) n^{3/2} A D_o^{1/2} v^{1/2} C_o$$

(Bard and Faulkner.[68])

where A is the electrode area in cm^2, D_o is the diffusion coefficient of O in cm^2s^{-1}, C_o is the concentration of ferricyanide in $mol.cm^{-3}$ and v is the scan rate in Vs^{-1}. Temperature of measurement = 25°C.

The diffusion coefficient of the species $Fe(CN)_6^{3-}$ has been accurately determined by von Stackelberg *et al*[69]. The above equation can thus be used to determine electrode surface area from a cyclic voltammogram of Potassium ferricyanide. (O = $Fe^{(III)}(CN)_6^{3-}$, R = $Fe^{(II)}(CN)_6^{4-}$)

It can be seen from the equation that the peak cathodic current i_{pc} is directly proportional to the electrode area.

Cyclic voltammograms were performed using the conditions stated above on a series of three electrodes (stored in RO water at 25°C), weekly for four weeks. During this time, measured peak cathodic currents from these electrodes varied by less than 1%.

3 WARFARIN

3.1 Introduction

3.1.1 Structure

Warfarin is 3-(α-acetonylbenzyl)-4-hydroxycoumarin. The structure of the sodium salt is shown in Figure 3.1.

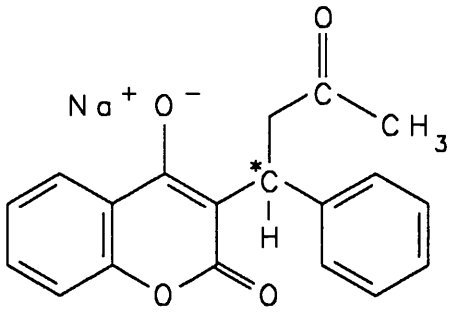


Figure 3.1 The structure of the sodium salt of warfarin showing the chiral centre *

The molecule has a chiral centre as indicated, and the commercially available preparations of warfarin consist of racemic mixtures.

The pK_a of warfarin is 5.0[100]

3.1.2 Actions and Uses

Warfarin is licensed in Great Britain for use as an oral anticoagulant and it is widely used in the treatment of, and prophylaxis against, thromboembolism.

In order to understand the mode of action of warfarin, it is necessary to consider the vitamin K cycle.

1) A reduced form of vitamin K is produced by the action of vitamin K reductase on vitamin K

2) The reduced form of vitamin K is involved as a cofactor in the activation of various clotting factors (II, VII, IX and X and the anticoagulant protein C) under the influence of γ-glutamyl decarboxylase. Vitamin K epoxide is produced during this reaction

3) Vitamin K is regenerated by the action of vitamin K epoxide reductase on vitamin K epoxide to complete the cycle.

The action of warfarin is thought to occur by the inhibition of vitamin K reductase and vitamin K epoxide reductase although exact mechanisms are still to be established[70,71,72].

3.1.3 Do we need a Biosensor for Warfarin

When administered, warfarin binds to plasma and hepatic proteins. Additionally, it undergoes a variety of metabolic conversions including reduction of the aliphatic ketone group and hydroxylations of the coumarin ring structure and the benzyl group. These metabolites are produced in the liver and in the main they have little pharmacological activity.

The dosage required to produce equivalent levels of anticoagulation is subject to considerable inter-patient variation. Because of this, the effects of warfarin are usually monitored by regular patient attendance at an hospital anticoagulant clinic. The clotting time of a blood sample (which can be expressed in a variety of ways) is measured.

It would be desirable to have a simple and reliable home test by which the patient could adjust their warfarin dosage. Because of a poor correlation between free (unbound) blood warfarin levels and the degree of anticoagulation, a warfarin biosensor blood assay would not provide such a test.

A warfarin biosensor could be useful in the study of warfarin metabolism.

From the point of view of biosensor research, the development of a sensor for warfarin is of interest because if some of the technical hurdles can be overcome, the technology should be of use in the development of other sensors.

3.1.4 Microbial Models of Mammalian Metabolism

Micro-organisms are capable of using many varied molecules as carbon sources. In addition, they are able to produce or make changes to many complex compounds. They are used for the production of drug molecules such as antibiotics and can also be used to modify a variety of substrates, the latter being particularly useful if the modification is difficult to perform by *in vitro* chemical methods.

Many types of bacteria and fungi are able to modify drug molecules in ways analogous to mammalian metabolic systems. In a review article in 1975, Smith and Rosazza[73] described systems which they termed "Microbial Models of Mammalian Metabolism". They showed that there were many examples in the literature of microorganisms which exhibited the ability to biotransform compounds in ways analogous to Phase 1 (pre-conjugation) reactions seen in mammals. Such microorganisms are being studied[74] with the aim that a range of cultures could be used to reliably predict the likely mammalian metabolites of new drug substances (e.g. Jurgens and Clark[75]), and that the cultures could be used to "synthesize" sufficient quantities of such metabolites for structure elucidation.

3.1.5 Warfarin Metabolism by Microorganisms

Warfarin has been used as a "metabolic probe" in the study of hepatic mixed function oxidases in various mammalian systems[76], and therefore much attention has been directed to finding micro-organisms which can metabolise warfarin in a similar manner to mammals.[77,78]

Davis and Rizzo[77] screened nineteen different microbial cultures for an ability to metabolise warfarin and found two (namely *Nocardia corallina* [ATCC 19070] and

Arthrobacter species [ATCC 19140]) which were able to perform the reduction of the aliphatic ketone group as the main transformation (Figure 3.2). The fungus *Cunninghamella bainieri* (UI-3065) has been shown to produce 4′-hydroxywarfarin as its main conversion product. Another of the *Cunninghamella* species, *Cunninghamella elegans* (ATCC 36112) has been shown to produce all the known hydroxylated metabolites of warfarin produced in mammalian systems[79].

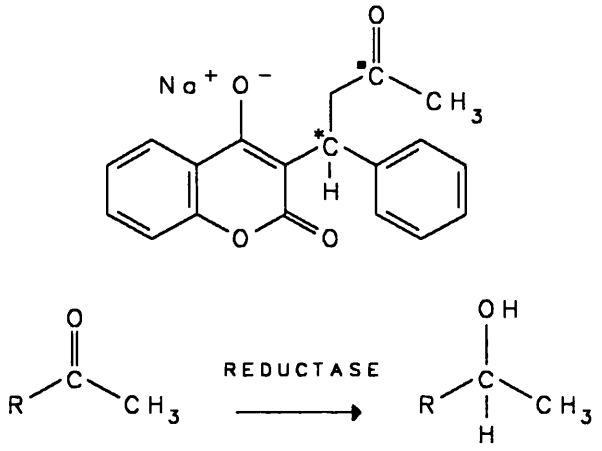


Figure 3.2 The structure of Warfarin Sodium showing the chiral centre (*). Also shown is the chiral centre created when the aliphatic ketone group (▪) is enzymatically reduced.

3.1.6 A Previously Attempted Warfarin Biosensor

Fitzgerald *et al*[80] attempted to create a biosensor for warfarin using the bacteria *Nocardia corallina* (which had been shown by Davis and Rizzo[77] to reduce the aliphatic ketone group of warfarin). This organism was immobilised at the surface of a glassy carbon rod together with ferrocene as an electron transfer mediator. The current flowing when a potential of 400mV (*vs* S.C.E.) was applied was proportional to the dissolved warfarin concentration (between 0.26 and 3.0×10^{-4} M.dm^{-3}) in a phosphate buffered saline solution.

By direct comparison with an amperometric enzyme electrode in which an oxidation is taking place (e.g. a glucose sensor based on glucose oxidase), if *Nocardia corallina* is

reducing the aliphatic ketone group of warfarin, one would expect to see the concomitant oxidation of perhaps an enzyme cofactor (cf reduction of FAD to $FADH_2$ in the glucose sensor) or the appearance of an oxidised co-substrate involved in the reaction (cf H_2O_2 appearance when $FADH_2$ is re-oxidised by dissolved oxygen). The electrode reaction would be expected to be a reduction (Figure 3.3), and the electrode potential would need to be more negative than the standard electrode potential for the reduction of the cofactor or co-substrate. It is therefore unclear what was happening in this biosensor. It would have been useful to have seen the current generated over the same concentration range of warfarin but from an electrode constructed in the same manner but without the inclusion of the bacteria. If inclusion of the bacteria was demonstrated to be necessary for the generation of the current, then it may have been that the electrode was detecting the products of other types of metabolic activity of the bacteria.

3.1.7 Specificity of biosensors based on Reductases

The question arises:- will a biosensor based on a ketone reductase be sufficiently specific. Many drug molecules contain a ketone group. Many microorganisms can be found with an ability to reduce ketones to alcohols. Not all of these however will reduce the ketone group found in warfarin and the ones that do are therefore exhibiting a degree of specificity.

In mammals several enzyme systems exist for the reduction of aldehydes and ketones. Liver alcohol dehydrogenase is able to reversibly catalyse the reduction of aldehydes and ketones to alcohols. The reduction of aldehydes by this enzyme system is relatively unimportant because there are enzyme systems which will oxidise aldehydes to carboxylic acids which can be readily excreted.

Reduction of ketones by alcohol dehydrogenase is an important biotransformation which increases the polarity of the compound and provides a site for phase 2 (conjugation) reactions.

The ease with which an alcohol dehydrogenase is able to reduce a ketone depends upon the bulkiness of groups attached to it. In addition, when the alcohol is produced, a chiral

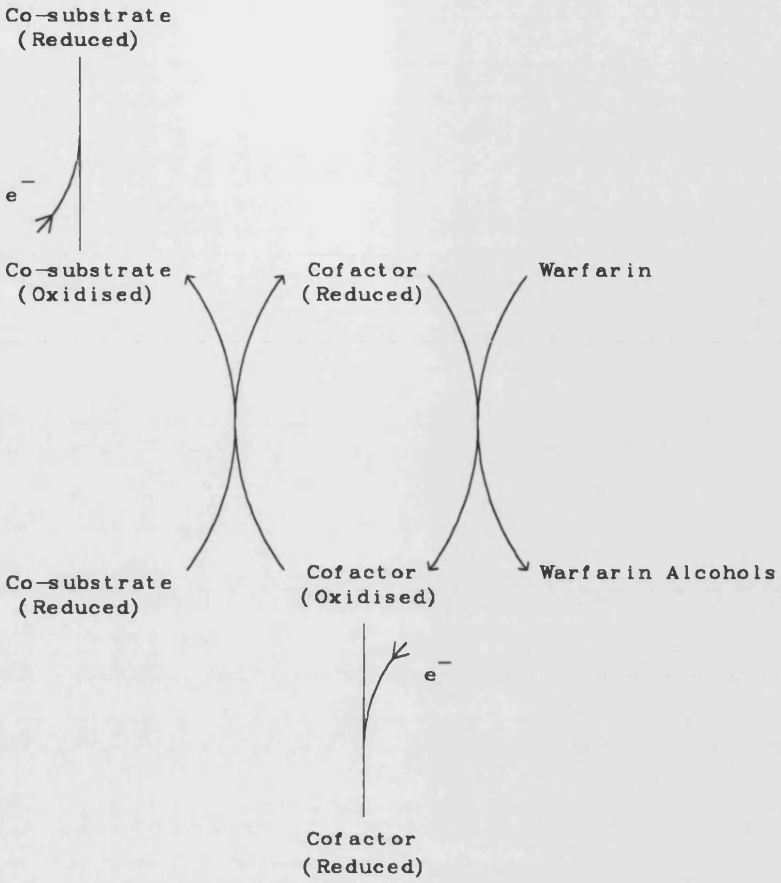


Figure 3.3 The sequence of events at a biosensor based on the detection of the reduction of warfarin

centre is formed. All commercially available alcohol dehydrogenases, from microorganisms and from mammalian liver extracts, reduce ketones to alcohols with same spatial configuration with respect to groups originally attached to the carbonyl group. Recently, Shen *et al*[81] have screened microorganisms and found a *Pseudomonas* species which is capable of producing alcohols with the opposite spatial configuration.

The reduction of warfarin to warfarin alcohols in cytosolic fractions from the liver and kidney from humans and from rats has been studied by Moreland and Hewick.[82] This study gave some important findings. Previous workers (cited in the above paper) had shown that in man, warfarin alcohols represent about 18% of the urinary metabolites of

warfarin compared to about 7% in rats. When R-warfarin alone was administered, the proportion of warfarin alcohols in human urine rose to 37% suggesting that S-warfarin is not significantly reduced to the alcohol in humans. As mentioned earlier, when the reduction occurs, a second chiral centre is created and two diastereomeric alcohols are produced. Each diastereomer exists as an enantiomeric pair. These can be described as:-

R-warfarin-S-alcohol and S-warfarin-R-alcohol

S-warfarin-S-alcohol and R-warfarin-R-alcohol

After R-warfarin administration the alcohol found in the plasma is almost entirely R-warfarin-S-alcohol. After S-warfarin administration the alcohol found in the plasma is mainly S-warfarin-S-alcohol. However, approximately five times as much warfarin alcohol was found in the urine after a single dose of R-warfarin when compared with a single dose of S-warfarin. This seemed to correlate with the product profile that would be expected from the reduction of warfarin by liver alcohol dehydrogenase, with the majority product being the S-alcohol.

Moreland and Hewick[82] showed that the enzyme responsible for the reduction was NADPH dependent (NADH being only one tenth as effective as a cofactor) and therefore it was not alcohol dehydrogenase but a separate distinct ketone reductase. It is possible that the low level of activity seen when only NADH was present may have been due to there being a mixture of alcohol dehydrogenase and the ketone reductase in their preparations.

These results have important implications for the specificity of potential biosensors based on ketone reductases. Although the reductase described by Moreland and Hewick is probably not specific purely for warfarin it would appear that it has a much higher activity on the ketone group in warfarin than alcohol dehydrogenase does.

The results of Apanovitch *et al*[83] are somewhat contradictory. They demonstrated the reduction of S-warfarin to the S-warfarin-S-alcohol (but little detectable R-warfarin reduction), and the reduction of the ketone group at the C20 position in progesterone (aliphatic) to the corresponding 20β-alcohol (R-Configuration), by an NADPH dependent

system in rat hepatic microsomes. They also showed differences in the activity of this ketone reduction system dependent upon the age, sex and strain of the rats used and suggested that one enzyme might be responsible for the reduction of both compounds. They also demonstrated NADH dependent reduction of progesterone to the 20α-alcohol (S-Configuration).

By isolating ketone reductases from a number of different sources it may be possible in the future to create a multi-biosensor probe. This would consist of several discrete ketone reductase electrodes each with its own degree of specificity for different ketone containing analytes. It may be possible for such a probe to actually identify a compound from the current profile produced, and then to measure its concentration from the electrode producing the highest current. It is as yet unknown of course whether ketone reductases from different sources will exhibit different specificities or not, or whether it will turn out to be a ubiquitous enzyme system. The results of the work of Shen *et al*[81] are encouraging in this regard.

3.1.8 Daunorubicin Reductase

When the literature was searched for other examples of the reduction of drug molecules which contain a ketone group in the liver, an interesting example came to light. The anti-cancer drug Daunorubicin contains an aliphatic ketone group (Figure 3.4) and is converted in the liver to the corresponding alcohol[84,85,86]. The closely chemically related compounds of doxorubicin, epirubicin and idarubicin are more widely used than daunorubicin (which is available in the United Kingdom on a named patient basis only). Although the stereochemistry is not described in these papers it was established that the enzyme responsible for the reduction was NADPH dependent.

Daunorubicin is an anthracycline antibiotic which inhibits DNA and RNA synthesis. It is isolated from *Streptomycetes peucetius*[71] and it may therefore be possible to find microorganisms which will grow in its presence and are able to reduce the aliphatic ketone group.

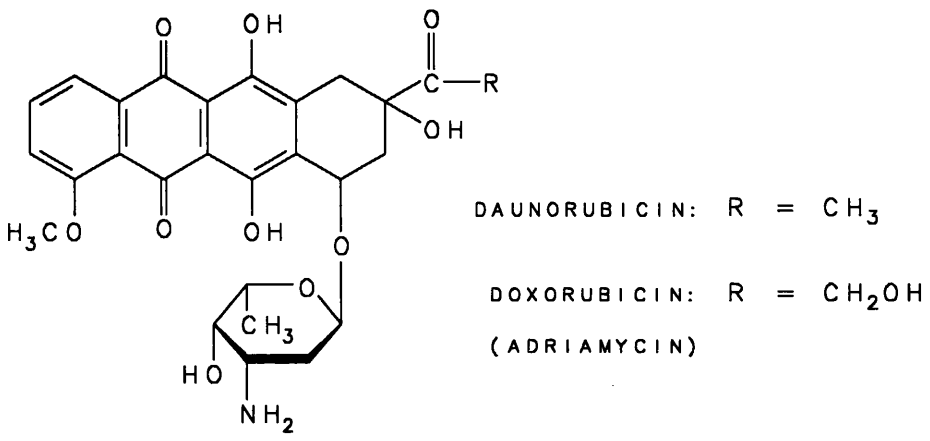


Figure 3.4 The structure of Doxorubicin and Daunorubicin

A biosensor for any of these compounds would be very useful as a research tool, and might also be clinically useful. This class of anti-cancer drugs exhibits a high degree of cumulative cardiotoxicity. Dosage is usually calculated on the basis of the surface area of the patient (which is a derived parameter calculated from a weight-height-surface area nomogram) but if a biosensor existed a more precise dosage determination might be possible from the results of measuring the plasma drug concentration-time profile during the first administration to the patient.

3.2 Experimental details

3.2.1 Equipment

Gilson model 116 variable wavelength UV detector

LDC Constametric 3000 dual piston metering HPLC pump (two)

BBC Servogor SE120 chart recorder or -

Servoscribe 1s chart recorder

HPLC column water bath - Grant SU6 re-circulating water bath heater running against a Haake EK12 water bath cooling coil.

Rotary evaporator, Büchi rotavapor

Mettler AE163 4 or 5 decimal place analytical balance

BBC model B microcomputer, Acorn Computers, England

Polarimeter, Optical Activity Ltd. England.

Perkin Elmer λ3B dual beam scanning UV/Visible spectrophotometer

Matched 1cm path length quartz cells for the above

3.2.2 Materials

3.2.2.1 Chemicals

Racemic warfarin 98% (free acid), potassium chloride 99+ % (ACS Reagent), phosphorous pentoxide 98+% (ACS reagent), sodium borohydride 98% and acetic acid 99.7% (ACS reagent) were obtained from Aldrich Chemical Company Ltd.

Hydrochloric acid solution, sodium hydroxide volumetric concentrates, quinine and quinidine (98%), magnesium sulphate (99+%) were obtained from BDH Chemicals Ltd., Poole, Dorset, England.

Chloroform and HPLC grade methanol, dichloromethane and heptane were obtained from Fisons scientific, Loughborough, England.

3.2.2.2 HPLC Column

Preparative column: 10μm particle size Zorbax Sil (Silica), 21.2mm internal diameter, 250mm length)

3.3 Resolution of Racemic Warfarin

In order to study the metabolism of warfarin by microorganisms it was preferable to be able to expose them to either pure S-warfarin or pure R-warfarin rather than a racemic mixture.

3.3.1 Method

Racemic warfarin was resolved according to the method developed by West *et al*[87]. This involves preferentially forming the salt of S-warfarin with the alkaloid quinidine and preferentially forming the salt of R-warfarin with quinine (the naturally occurring enantiomer of quinidine).

The structure of quinine/quinidine is shown below (Figure 3.5).

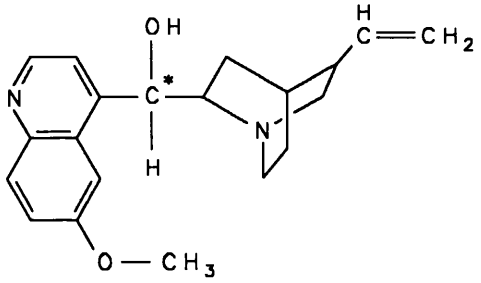


Figure 3.5 The structure of quinine/quinidine showing the chiral centre *

3.3.1.1 S-warfarin

This first section of the method was performed at 13/100 scale when compared to the above paper and is summarised below. Intermediate optical rotations were not performed.

Solution 1: 40g warfarin (0.13 mole) and 42.4g quinidine were dissolved by warming in 650ml of solution consisting of 40% chloroform in acetone. This solution was then held at —10°C until crystals of S-warfarin quinidine were formed (36 hours).

The temperature of —10°C was achieved by running a water bath cooling coil against a water bath heater in a 4°C refrigerated room. The solution in the water bath consisted of distilled water containing approximately 30% of a commercially available automotive antifreeze solution. The water bath temperature was monitored using a thermistor based sensor linked to a BBC microcomputer which was used to switch the water bath heater on or off as appropriate. The details of the circuitry for this are given in reference [88]

The solution was filtered and the filtrate was saved for later.

Solution 2: The crystals of S-warfarin quinidine were redissolved in 450ml of gently warmed acetone. This solution was then cooled to 0°C using the same equipment described above. The crystals formed were collected by filtration and the filtrate was discarded.

Solution 3: The recrystallised S-warfarin quinidine collected from solution 2 was dissolved in 65ml of chloroform. This solution was then extracted by shaking with 130ml of 0.5M Sodium Hydroxide solution in a separating funnel. This stage of the process liberated free quinidine, which remained in the chloroform layer, and formed the sodium salt of S-warfarin which partitioned into the aqueous layer.

After separation of the aqueous layer, S-warfarin (free acid) was precipitated by the addition of excess 1M Hydrochloric acid. The free acid was then extracted from the solution by shaking with three 40ml aliquots of chloroform.

The chloroform layers were combined and the solution dried, firstly by shaking with 30ml of saturated potassium chloride solution which was then removed using a separating funnel, and secondly by shaking the solution with 3g of dried magnesium sulphate powder which was then removed by filtration through a cotton wool plug.

The resulting solution was then evaporated to dryness using a rotary evaporator.

Solution 4: The S-warfarin powder was dissolved in 78ml of warmed solution consisting of 80% acetone in water.

Unfortunately, at this stage crystals did not form on cooling. The solution was taken back to dryness in a rotary evaporator then the powder was dissolved in a minimum amount of warmed 50% acetone in water (60ml). The crystals formed on cooling were collected by filtration (S-warfarin Crop 1).

A further 13mls of water was added to the filtrate which caused it to become cloudy. The solution was gently heated until it became clear. The second set of crystals formed on cooling were again collected (Crop 2). A further 13mls of water was added and the procedure repeated. (Crop 3)

3.3.1.2 R-warfarin

The filtrate from section 1 (above) was concentrated to approximately 65ml and was diluted with an equal volume of acetone upon which the solution became cloudy. Gentle warming was used to redissolve the solid. On cooling a precipitate was formed which was removed by filtration. The filtrate was evaporated to dryness in a rotary evaporator, forming a glass.

Solution 5: The glass was redissolved in 130ml of chloroform and extracted using 260ml of 5% aqueous sodium hydroxide solution. The aqueous layer was taken and added dropwise to 70ml of 5.65M aqueous hydrochloric acid solution forming a precipitate of partially resolved R-Warfarin which was removed by filtration.

This powder was dried (over phosphorous pentoxide in a vacuum oven at 40°C) to a constant weight of 17.93g (0.058 mole). At this stage of the resolution, West *et al*[87] had 124g (0.405 mole) of powder which when scaled down to the quantities used above would represent the equivalent of 16.12g. From this point on, the quantities of chemicals used by West *et al* were multiplied by a factor of 0.143 (0.058 ÷ 0.405).

Solution 6: 17.93g of the partially resolved warfarin (0.058 mole) was added to a boiling solution consisting of 122ml of absolute ethanol containing 18.87g of quinine (0.058 mole). Following the cooling of the solution to room temperature, 480ml of dry ether was added and the solution was cooled further and held at —10°C (as before) until crystallisation had occurred (24 hours).

The R-warfarin quinine was collected by filtration which was carried out as quickly as possible in the 4°C cold room.

This product was recrystallised twice by dissolving in 3ml per gram of hot absolute ethanol, then adding 12ml per gram of dry ether to the cooled solution, followed by cooling to —10°C. A final crystallisation was performed by dissolving in 2.5ml per gram of hot absolute ethanol and allowing to cool slowly to room temperature.

Solution 7: The recrystallised R-warfarin quinine collected from solution 6 was dissolved in 140ml of chloroform. This solution was then extracted by shaking with 140ml of 5% sodium hydroxide solution in a separating funnel. This stage of the process liberated free quinine, which remained in the chloroform layer, and formed the sodium salt of R-warfarin which partitioned into the aqueous layer

The aqueous layer was then added dropwise to 70ml of 5.65M aqueous hydrochloric acid solution forming a precipitate R-Warfarin which was removed by filtration.

Solution 8: The R-warfarin was dissolved in a minimum amount of warm 50% aqueous acetone solution and allowed to cool. The crystals formed were collected by filtration (R-warfarin crop 1). The filtrate had more water gradually added until it became cloudy. It was then warmed until the solution became clear. Further crystals formed on cooling to room temperature (crop 2).

3.3.1.3 Optical Activity, Melting Point and Assay

The optical rotation produced by each crop of crystals was measured at the same concentration and in the same solvents as in the paper by West *et al*[87] for comparison.

The melting point of each sample was measured.

3.3.2 Results

Sample	Yield (g)	Specific rotation $[\alpha]_D^{25}$		Melting Point (°C)
		Measured	Ref. 87	
S-warfarin 1st crop	3.39	-148.9	-148.0	171-172
S-warfarin 2nd crop	2.01	-146.8	-148.0	171-173
S-warfarin 3rd crop	1.53	-47.9	---	160-164
R-warfarin 1st crop	4.80	+147.3	+149.0	171-172
R-warfarin 2nd crop	0.52	+97.2	---	155-160

Measurements were performed as solutions in sodium hydroxide solution 0.5M.dm^{-3}.

S-warfarin solutions analysed contained 1.2% and R-warfarin solutions contained 2% warfarin.

Ultraviolet/visible absorption spectra and HPLC chromatograms of S-warfarin crops 1 and 2, and of R-warfarin crop 1 (0.0015% solutions in methanol) were indistinguishable from each other or from that of racemic warfarin. Single peaks were seen on the HPLC system described in chapter 4.

Following these results, S-warfarin crops 1 and 2 were combined. R-warfarin crop 2 and S-warfarin crop 3 were not used.

3.4 Chemical synthesis of warfarin alcohols

In order to provide positional markers for the presence of "warfarin alcohols" (from the reduction of the ketone group) during the HPLC analysis of growth media, the alcohols were synthesised.

3.4.1 Method

The method used sodium borohydride to perform the reduction as outlined by Trager *et al*[89].

1g of racemic warfarin (3.24×10^{-3} mole) was dissolved in 25ml of 0.132M sodium hydroxide solution. 100mg (2.64×10^{-3} mole) of sodium borohydride was added and the solution was allowed to react at room temperature (with magnetic stirring).

0.1ml samples were taken during the reaction and acidified with 0.3ml of 0.1M hydrochloric acid solution followed by dilution to 10ml with HPLC grade methanol. This solution was further diluted 1 in 10 with mobile phase (see HPLC methodology in chapter 4) and analysed using HPLC. The reaction was allowed to continue until the peak representing the residual warfarin had fallen to below 1% of its value at t_0. This took 270 minutes.

Example chromatograms from the start of the reaction (Figure 3.6) and run during the reaction (Figure 3.7) are shown below.

On completion of the reaction, 0.1M hydrochloric acid was added dropwise until all the sodium borohydride had decomposed (as indicated when drops of acid no longer cause the liberation of bubbles of hydrogen). It was then added in excess in order to precipitate the warfarin alcohols.

The product was extracted by shaking with three 25ml aliquots of diethyl ether in a separating funnel. The etherial layers were combined and dried by shaking with 20ml of saturated potassium chloride solution. The aqueous layer was then removed using a separating funnel.

The etherial layer was dried further by shaking with approximately 4g of dried magnesium sulphate powder, which was then removed by filtration through a cotton wool plug. The mixture was evaporated to dryness in a rotary evaporator.

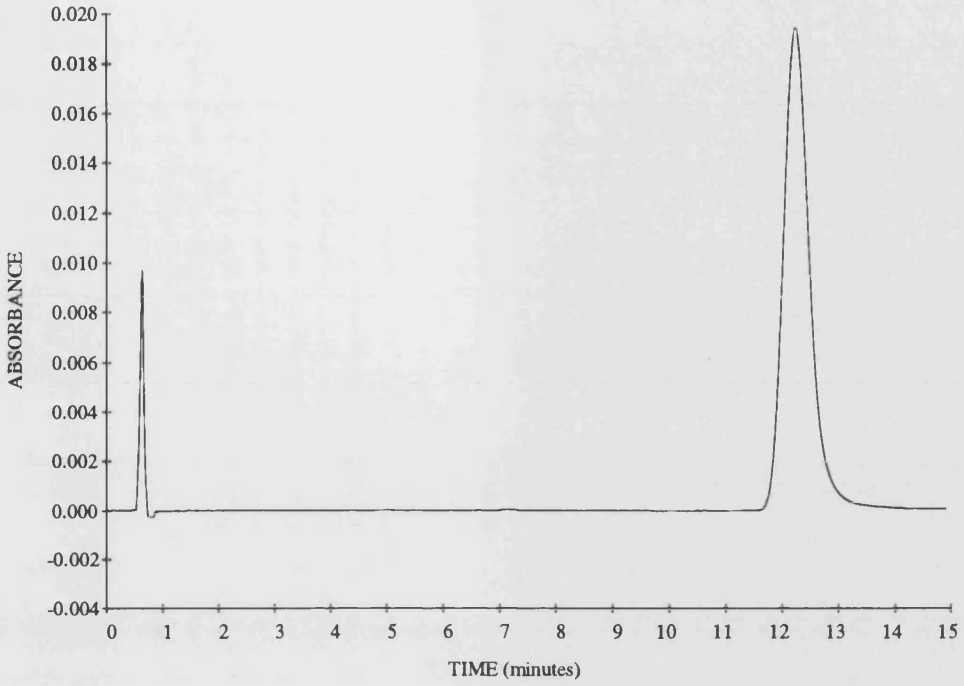


Figure 3.6 Chromatogram of the Warfarin starting solution prior to the addition of sodium Borohydride

3.5 Separation of the alcohols

It was hoped that the conversion of warfarin to the corresponding alcohols by the microorganisms (Chapter 4) would have been high enough for the process to be followed by looking at the decrease in warfarin in the growth media. This did not turn out to be the case, and it became apparent that it was desirable to obtain pure samples of the alcohols to act as standards.

This method for the preparative separation of the two enantiomeric pairs of alcohols was not developed in time for them to be used as analytical standards for the work in this thesis. It is included because it is significantly easier than the elution chromatography method developed by Chan *et al*[90].

The majority of analytical HPLC methods in the literature have used reversed phase systems. However, there have been a number of normal phase systems.

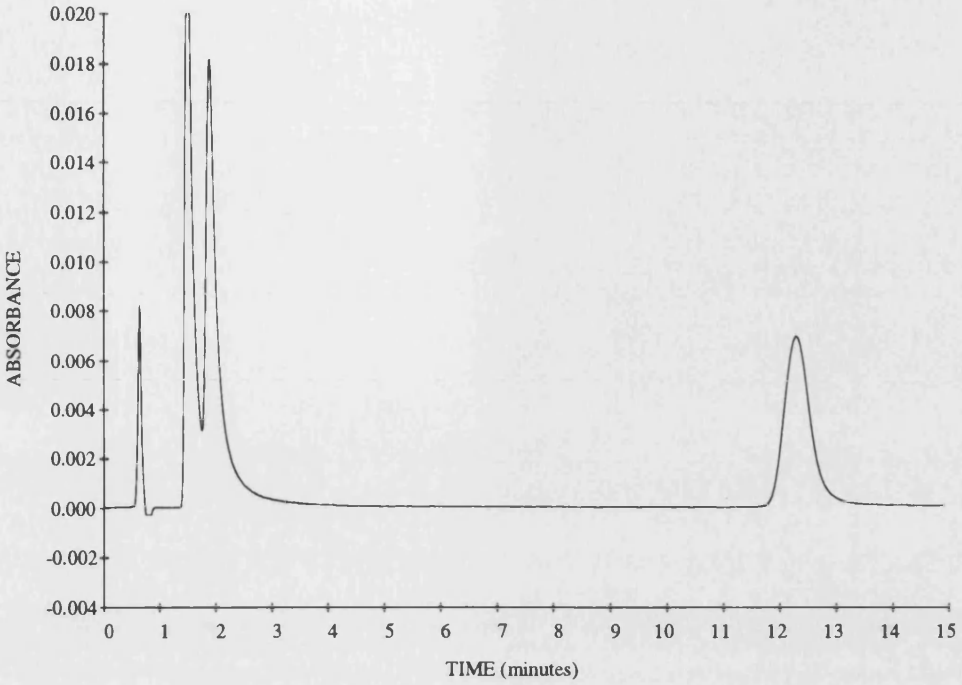


Figure 3.7 Chromatogram performed during the reduction of warfarin by sodium borohydride (45 minutes sample)

Lee *et al*[91] used a mobile phase consisting of 75% Heptane with 25% (79.3% dichloroethane, 20% ethyl acetate, 0.7% acetic acid) with a silica column with a bonded cyano/amino moiety (Partisil). They also pointed out that there was little difference between this and a plain silica column.

Banfield and Roland have used two slightly different systems; hexane/ethyl acetate/methanol/acetic acid 74.75/25/0.25/0.3[92] and 74.75/25/0.25/0.4[93] on plain silica columns but with derivatisation of the sample prior to analysis with the second system.

A variety of solvent systems have been able to demonstrate separation of the alcohols on plain silica thin layer chromatography (TLC) plates[77]. The amount of acetic acid in the solvent system seems to be an important factor in achieving separation.

3.5.1 Method

Although the optimisation of a four component solvent system is difficult to achieve, a mobile phase that worked for normal phase HPLC and was based on a combination of those used above was arrived at empirically.

Solution A consisted of dichloromethane/methanol/acetic acid in the ratio 30/10/2 by volume. Solution B was heptane. The combination of these which gave the desired separation was 10% A and 90% B.

Solution A had dichloromethane and methanol in approximately the same ratio as in the paper by Lee *et al*[91] but had a higher concentration of acetic acid, bringing the overall concentration in the mobile phase to 0.476%.

The separations were carried out at room temperature using a plain silica preparative column (21.2mm internal diameter × 25cm containing Zorbax Sil, 10μm particle size), using ultraviolet detection at 305nm. Two HPLC pumps, each capable of providing 10ml.min^{-1} of output, were used to provide the high flow rate required. A measured flow rate of 20.5ml.min^{-1} was achieved with a back pressure of approximately 2800 p.s.i.

The sodium borohydride reduced warfarin sample was introduced onto the column as a 200mg.ml^{-1} solution in solvent system A. A 0.5ml loop size was used, leaving to a column loading of 100mg.

3.5.2 Results

The chromatogram shown in Figure 3.8 shows almost baseline separation of residual warfarin (30 minutes), R-warfarin-S-alcohol and S-warfarin-R-alcohol (38 minutes) and R–warfarin-R-alcohol and S–warfarin-S-alcohol (51 minutes).

Peak assignments given are based on the reverse of the elution order given in the paper by Fasco *et al*[76]. Samples of the alcohols collected from this system were chromatographically pure when run on the reverse phase HPLC system described in chapter 4.

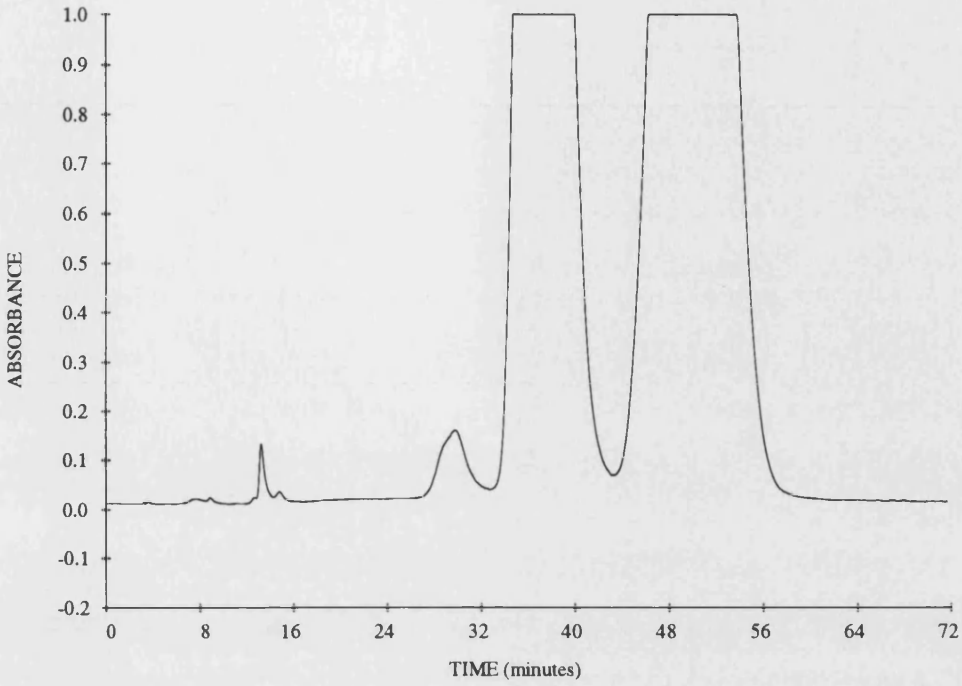


Figure 3.8 The preparative HPLC separation of the warfarin alcohols

4 MICROORGANISMS

4.1 Introduction

Several microorganisms which have been reported in the literature to metabolise warfarin were obtained and were screened with the aim of re-establishing warfarin metabolism in our own laboratories. A range of other related microorganisms, which were held in the University of Bath pharmacy department stocks were also screened.

It was intended to attempt to produce large quantities of any microorganism shown to produce reduction of the ketone group of warfarin, by using a fermenter.

4.2 Experimental Details

4.2.1 Equipment

LDC Spectromonitor 3100 variable wavelength UV detector

LDC Constametric 3000 dual piston metering HPLC pump

BBC Servogor SE120 chart recorder or -

Servoscribe 1s chart recorder

Hewlett Packard 3390A integrator with integral printer

HPLC column water bath - Grant SU6 re-circulating water bath heater running against a Haake EK12 water bath cooling coil.

Chilspin Bench centrifuge - Fisons Scientific Equipment, Loughborough, England

Bioflow II Batch or continuous 6l Fermenter and Controlled environment incubator/shaker - New Brunswick Scientific, Edison, N.J., USA.

8 Flask shaker - Griffin and George, England

4.2.2 Materials

4.2.2.1 Chemicals and growth media

All salts used to make the chemically defined media were of standard laboratory reagent grade or above. All complex growth media were obtained from Oxoid, Basingstoke, Hants,

England and were reconstituted according to the manufacturers instructions using laboratory distilled water.

HPLC grade Acetonitrile and ethyl acetate were obtained from Fisons Scientific Equipment, Loughborough, England.

Trizma (Tris) buffer salts were obtained from Sigma Chemical Co. Ltd. Poole, Dorset

4.2.2.2 HPLC Column

Jones Chromatography 5µm particle size spherisorb ODS 2, 5mm internal diameter, 150mm length

4.2.2.3 Other Items

0.2µm cellulose nitrate 25mm diameter filters were obtained from Whatman Ltd., Maidstone, England

4.3 Reverse phase HPLC analysis of warfarin

In order to screen various micro-organisms for the ability to reduce warfarin's aliphatic ketone group to the corresponding alcohol it was first necessary to set up an assay for these compounds. The reversed phase HPLC system developed by Fasco *et al*[76] was used initially.

4.3.1 Preparation of solutions for HPLC

4.3.1.1 Mobile phase

The mobile phase composition was the same as the one used by Fasco *et al*[76] and consisted of:- 69 parts: 1.5% acetic acid solution in RO water (adjusted to pH 4.7 using concentrated ammonium hydroxide)

31 parts: HPLC grade acetonitrile

4.3.1.2 Racemic warfarin

For the initial calibration run racemic warfarin was dissolved in methanol/acetonitrile (1:1). The resulting solutions were then diluted 1 in 4 with mobile phase. This dilution procedure mimicked the dilution which occurred during preparation on samples extracted from the microorganisms growth media.

4.3.2 HPLC methodology

A water bath was used to keep the HPLC column temperature at 25°C. The mobile phase flow rate was 2.0 $ml.min^{-1}$ and a 50µm sample injection loop was used. Ultraviolet detection at a wavelength of 305nm was employed.

4.3.3 Calibration Curves

In order to check the correct functioning of equipment, simple five point calibration curves were performed (two injections at each point). A typical curve is given below (Figure 4.1)

This curve was constructed using dilutions prepared from one warfarin weighing. This demonstrated the linearity of the assay.

In order to assess the repeatability of the analysis system, five solutions of around 0.004% were prepared together with corresponding 1 in 10 dilutions. Each solution was injected onto the column twice, or until integrator peak areas were within 2% of each other (usually only two injections).

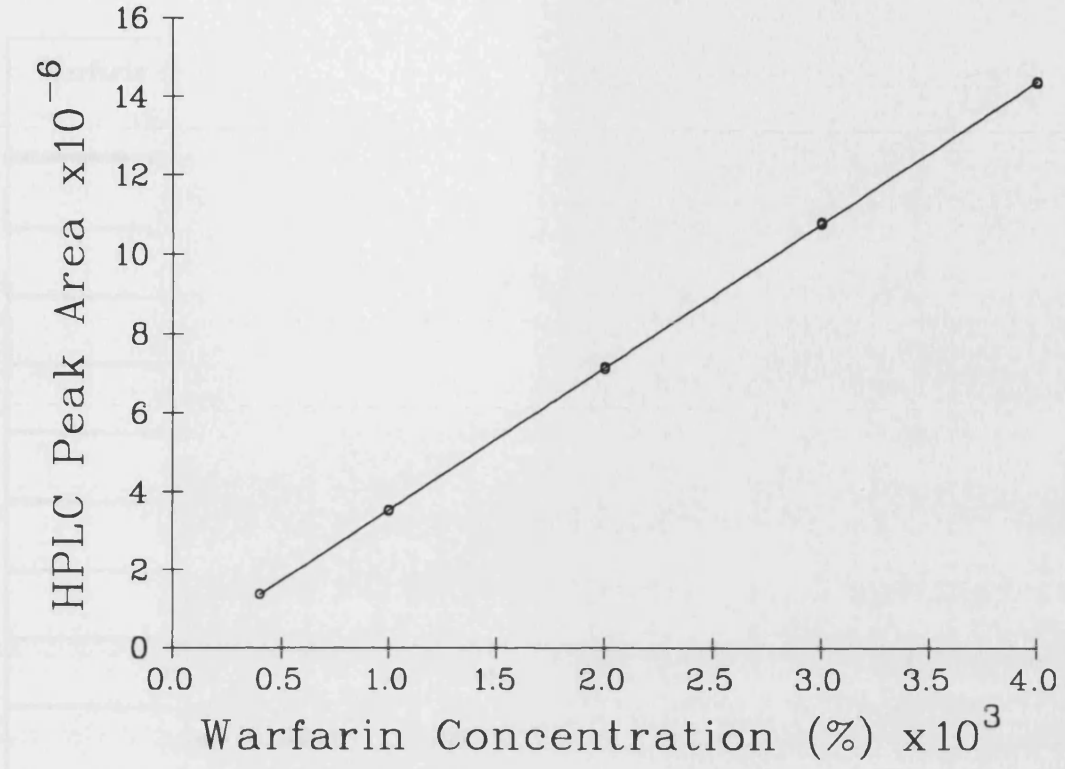


Figure 4.1 HPLC calibration curve for warfarin

Warfarin Concentration (% × 10^3)	Peak Area (× 10^{-7})	Peak Area of a 1 in 10 dilution (× 10^{-6})
3.965	10.546	9.9191
3.965	10.598	10.089
4.015	10.524	10.146
4.015	10.574	10.092
3.970	10.460	9.8786
3.970	10.529	9.8440
4.065	10.794	9.9436
4.065	10.825	10.080
3.955	10.384	9.9569
3.955	10.424	9.8753
Statistics from peak area ÷ concentration (× 10^{-10})		
Minimum	2.621	2.446
Maximum	2.673	2.545
Mean	2.645	2.500
Standard Deviation	0.0175 (0.66% of the mean)	0.0280 (1.12% of the mean)

At each dilution, the solution which gave the peak area/concentration value nearest to the mean value was then used as a standard.

The assay of extracted samples was performed according to the following protocol.

High standard injected twice

Sample A injected twice

Sample B injected twice

Low standard injected twice

Sample C injected twice

Sample D injected twice

High Standard injected twice

Sample E injected twice etc.

The concentration of warfarin in the unknown samples could then be determined.

4.4 The screening of microorganisms for warfarin reductase activity

The following microorganisms were screened for warfarin reductase activity:-

FUNGI

Cunninghamella bainieri	C43
Cunninghamella bertholletiae	C1
Cunninghamella blakesleeana	CMI 53585
Cunninghamella echinulata	IMI 199844
Cunninghamella echinulata	C7
Cunninghamella echinulata	NRRL 3655
Cunninghamella elegans	ATCC 36112

The top six of these had previously been used in studies on the N-dealkylation of drugs at the University of Bath by Sewell[94].

Sources:-

C	American Cyanamid Company, Lederle Laboratories, Pearl River, New York, USA.
CMI/IMI	Commonwealth Mycological Institute, Kew, Surrey, England

NRRL U.S. Department of Agriculture Northern Regional Research Labs, Peoria, USA.

ATCC American Type Culture Collection, Maryland, USA

BACTERIA

Rhodococcus	ATCC 19140	(*Arthrobacter* species)
Rhodococcus	ATCC 19070	(*Nocardia corallina*)

4.4.1 Nomenclature

The classification of the *Nocardia Corallina* and *Arthrobacter* species referred to by Davis and Rizzo[77] has recently been changed. They are now considered to be of the genus *Rhodococcus*. For a full description of the properties which are required for this classification (mainly based on the cell wall composition) see Finnerty[95] and Topley and Wilson[96]. Because of this confusion in the literature, the ATCC numbers will be used in this text.

4.4.2 Screening protocol

A two stage protocol was used, based on the use of chemically defined media used by Eady *et al*[97] and modified by Gibson[98] and Sewell[94]

4.4.2.1 Culture maintenance

Fungal cultures were maintained on refrigerated (4°C) malt extract agar slants and were transferred to fresh slants every three months to maintain viability. Bacterial cultures were maintained on tryptone-soya agar slants in a similar manner.

4.4.2.2 Stage One Media

The mineral composition of the stage 1 basal media (at single strength) is shown below:-

Compound	Concentration (mg l^{-1})
$Na_2HPO_4.12H_2O$	989.0
$(NH_4)_2SO_4$	667.0
KH_2PO_4	378.0
Disodium EDTA	66.7
Na_2SO_4	56.0
$MgSO_4.7H_2O$	28.0
$ZnSO_4.7H_2O$	22.0
$FeSO_4.7H_2O$	11.10
$NaMoO_4.2H_2O$	5.60
$CaCl_2.2H_2O$	5.60
$MnSO_4.7H_2O$	2.20
$CuSO_4.5H_2O$	0.56

This solution was prepared at double strength. 100ml bottles of this solution and 100ml bottles of distilled water were sterilised by autoclaving.

A 5% solution of the Casamino acid (mixed amino acids) powder in distilled water and a 10% solution of glucose in distilled water were prepared. These solutions were sterilised by filtration through sterile 0.2µm pore size cellulose acetate membrane filters into sterile 20ml plastic vials.

The vessels used for both stage 1 and stage 2 cultures were thick walled, wide necked Erlenmeyer flasks which had six baffles moulded radially into the glass. They were pre-sterilised in a hot air oven.

Each flask had the following added aseptically:-

Double strength stage 1 basal media	25ml
5% Amino acid solution	10ml
10% Glucose solution	5ml
Distilled water	10ml

After equilibration of the solution to the incubation temperature the flasks were inoculated with surface growth from the slants. They were sealed using pre-sterilised bungs which consisted of a cotton wool ball which had been wrapped in gauze. The purpose of this was to allow a degree of oxygen diffusion into the flasks whilst maintaining sterility.

The flasks were incubated at 27°C in a controlled environment incubator/shaker running at 250 r.p.m. Cultures were incubated in the dark.

All fungal cultures grew well in this media, forming pellets of growth by approximately 36 hours from the time of inoculation.

The bacterial cultures grew fairly slowly on this medium but an adequate suspension of cells was produced by 48 hours.

4.4.2.3 Stage Two Media

The stage two media was similar to the stage one media but with the addition of further trace elements.

The composition of single strength solutions are given below:-

Stage two basal media

Compound	Concentration (mg l^{-1})
KH_2PO_4	3327.0
$NaH_2PO_4.2H_2O$	732.0
$(NH_4)_2SO_4$	500.0
$MgSO_4.7H_2O$	290.0
Disodium EDTA	290.0

Trace element solution

Compound	Concentration (mg l^{-1})
$(NH_4)_6Mo_7O_{24}.4H_2O$	93.0
$CaCl_2.2H_2O$	33.0
$ZnSO_4.7H_2O$	5.5
$FeSO_4.7H_2O$	3.5
$MnSO_4.4H_2O$	0.75
$CuSO_4.5H_2O$	0.20
$Co(NO_3)_2.6H_2O$	0.125
$Na_2B_4O_7.10H_2O$	0.090

The stage 2 basal media solution was again prepared at double strength and sterilised in an autoclave. The trace element solution was prepared as 50x strength concentrate and was sterilised by filtration.

Each stage 2 flask has the following added aseptically:-

Double strength stage 2 basal media		25ml
5% Amino acid solution		10ml
10% Glucose solution		5ml
50x strength trace element concentrate		1ml
0.5% warfarin solution (see below)		5ml
Solution from stage 1 flask*		1ml
Distilled water	to	50ml

* In the case of the fungal cultures, the inoculum from the stage on flask consisted of a single pellet. The volume of this was ignored.

The stage two flasks were set up using stage one cultures which were 72 hours old. The inoculum was the last solution to be added to the stage two flasks, after the other solutions had been pre-incubated for 1 hour to allow for temperature equilibration.

4.4.2.4 Preparation of the warfarin solution

These initial studies were carried out using racemic warfarin and the 0.5% solution was prepared as follows:-

1g (3.24 millimole) of warfarin powder was accurately weighed and was dissolved in 25ml of 0.132M sodium hydroxide solution (3.3 millimole), forming the sodium salt. A 0.1M hydrochloric acid solution was then added dropwise until the slightest cloudiness was seen in the solution. Distilled water was then added to 200ml, upon which the solution became clear again.

The pH of such a solution was measured and found to be 9.5.

In order to check that the stage two media was able to buffer the addition of this solution, two solutions were prepared:-

	Measured pH
1) 50ml stage two media including 5ml 0.5% warfarin	7.36
2) 50ml stage two media with 5ml of water replacing the warfarin	7.35

4.4.2.5 Controls

The following flasks were set up as controls:-

1) Stage two media with warfarin but no microorganisms. This was used to check for chemical degradation of warfarin during the incubation period.

2) Stage two media with each of the microorganisms but without warfarin. These flasks were used to check that the microorganisms were able to grow in the stage two media, and that they did not produce any compounds which interfered with the assay.

All flasks (stage one and stage two) were checked for contamination when they were finished with by preparing two tryptone-soya-agar streak plates and incubating one plate at 27°C and one plate at 37°C. If any contamination was apparent the experiment was repeated

4.4.2.6 Extraction Procedure

For this initial screening experiment, the following semi-quantitative extraction procedure was used:-

1.0ml of the growth media was transferred to a glass culture tube and was acidified with 0.1ml of 5.65 M aqueous hydrochloric acid solution. 4.0ml of ethyl acetate (HPLC grade) was added and the solution was mixed for 15 minutes using a flask shaker. The tube was then spun at 4000 r.p.m. in a bench centrifuge (Chilspin) for 5 minutes to aid separation of the organic layer. 3.0ml of the ethyl acetate solution was then transferred to a 10ml class A volumetric measure, and evaporated to dryness using a gentle stream of filtered compressed air. The residue was then redissolved by adding 2.5ml of a HPLC grade methanol/acetonitrile (1:1) mixture, and the flask was made up to volume using mobile phase.

4.4.2.7 Results

The second stage cultures were followed for a period of sixteen days. None of the fungal cultures showed any metabolism of warfarin to the corresponding alcohols. Both strains of *rhodococcus* showed some production of the warfarin alcohols (estimated at less than 5% conversion) with the first of the two alcohol peaks (S,S and/or R,R warfarin alcohols)

predominating. It had been hoped that this assay would be able to track the disappearance of warfarin from solution but the levels of conversion to the alcohol were too low for this.

4.4.3 Improving the assay

A method for improving both the separation of the two warfarin alcohols was sought together with a suitable compound for use as an internal standard.

4.4.3.1 A different mobile phase

The mobile phase used by Tasker[99] was tried. This consisted of 1% acetic acid and 44% acetonitrile in water. This gave baseline separation of the two pairs of warfarin alcohols and warfarin. In addition, the total assay time was reduced.

Acetic acid has a pK_a of 4.8[100] and a 1% solution (in water alone) has a calculated theoretical pH of around 2.8. Such a low pH was likely to cause damage to the analytical column, and therefore an old ODS column was placed in line to act as a guard.

4.4.3.2 Internal Standard

Davis and Rizzo[77] had used phenprocoumon as an internal standard, but this compound was not readily available. Two possible commercially available compounds for use as internal standards were DL–3–(α–acetonyl–p–chlorobenzyl)–4–hydroxycoumarin (subsequently referred to as chlorowarfarin), and the compound coumarin itself. When chlorowarfarin was injected onto the column using the HPLC system first described it had a retention time approximately twice that of warfarin. Coumarin, on the other hand, came off the column with the solvent front, but with the new mobile phase described above there was a significant delay before the peak. Also with this system, the coumarin peak (which comes off before the warfarin alcohols) was separated from any other peaks.

A chromatogram taken during the sodium borohydride reduction of a second batch of warfarin is shown in Figure 4.2. Peak identities:- R–warfarin-R-alcohol and S–warfarin-S-alcohol (5 minutes); R-warfarin-S-alcohol and S-warfarin-R-alcohol (7 minutes); residual

warfarin (9.3 minutes). Note: coumarin had a retention time of approximately 3 minutes on this system.

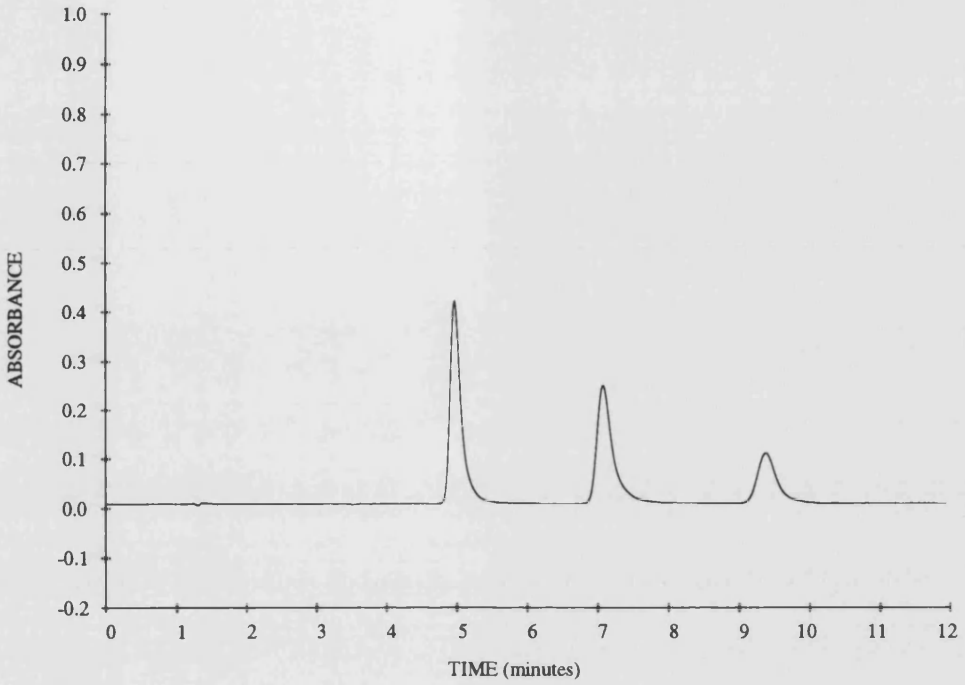


Figure 4.2 Chromatogram performed during the reduction of warfarin by sodium borohydride (120 minutes sample - second mobile phase)

4.4.4 Validation of the extraction procedure

It was found that both *rhodococci* grew more vigorously on the complex media tryptone soya broth (TSB). The extraction of warfarin from this media was attempted, together with the addition of coumarin as an internal standard during the extraction procedure. The extraction procedure was also modified in order to increase the final concentration of compounds in the injection sample.

Seven solutions of warfarin (as the sodium salt) in TSB were prepared covering the concentration range 0.006% to 0.06%. 1.0ml samples of each were placed in glass culture tubes and the following were added; 100µl of a 0.24% solution of coumarin in ethyl acetate,

100µl of 5.65M hydrochloric acid, 3.9ml of ethyl acetate. This solution was agitated using a flask shaker for a period of 5 minutes, followed by centrifuging at 4000 r.p.m. for 5 minutes to separate the aqueous and organic layers. 3.0ml of the supernatant was then evaporated to dryness. The residue was then dissolved by adding 0.5ml of methanol followed by 1.5ml of mobile phase. These samples were injected onto the column in a randomised order.

4.4.4.1 Results

The peak area ratio (warfarin peak area ÷ coumarin peak area) has been plotted against warfarin concentration in the TSB solution in Figure 4.3

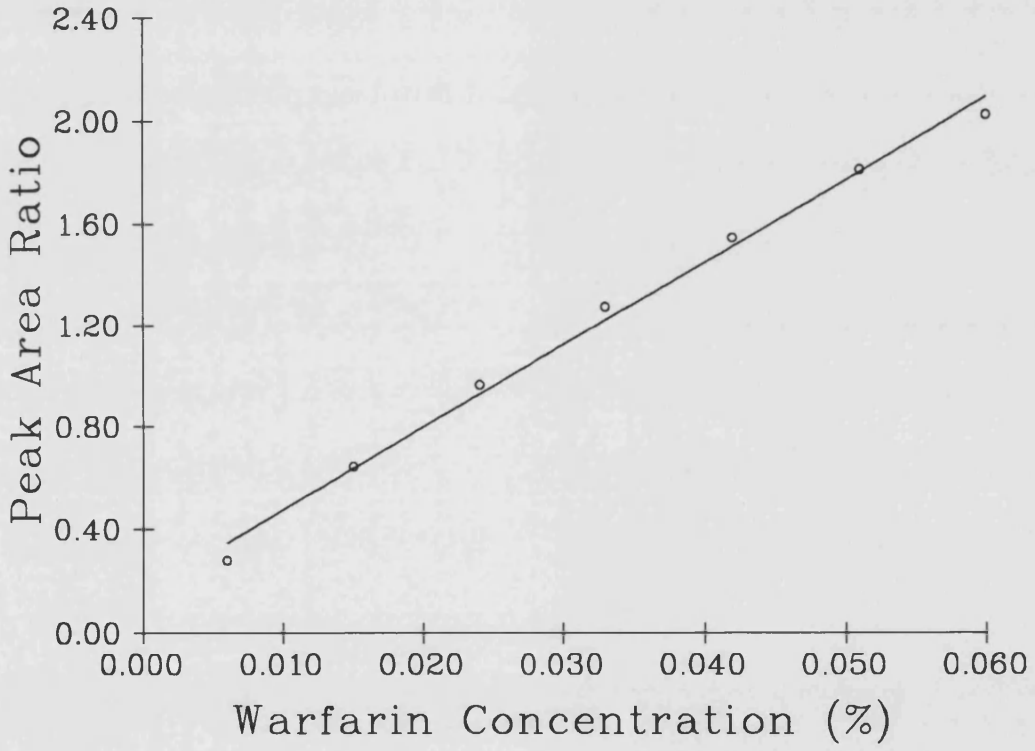


Figure 4.3 Peak area ratio *vs* warfarin concentration calibration graph (linear regression line shown)

STATISTICS

r-squared	0.99886	Correlation	0.99694
Calculated Intercept	0.152	Standard Deviation	0.0426
Calculated Slope	32.357	Standard Deviation	1.134

From looking at this data, it is obvious that although the correlation is reasonable, the intercept is considerably above zero. The same data, but with a non linear regression line is shown in Figure 4.4

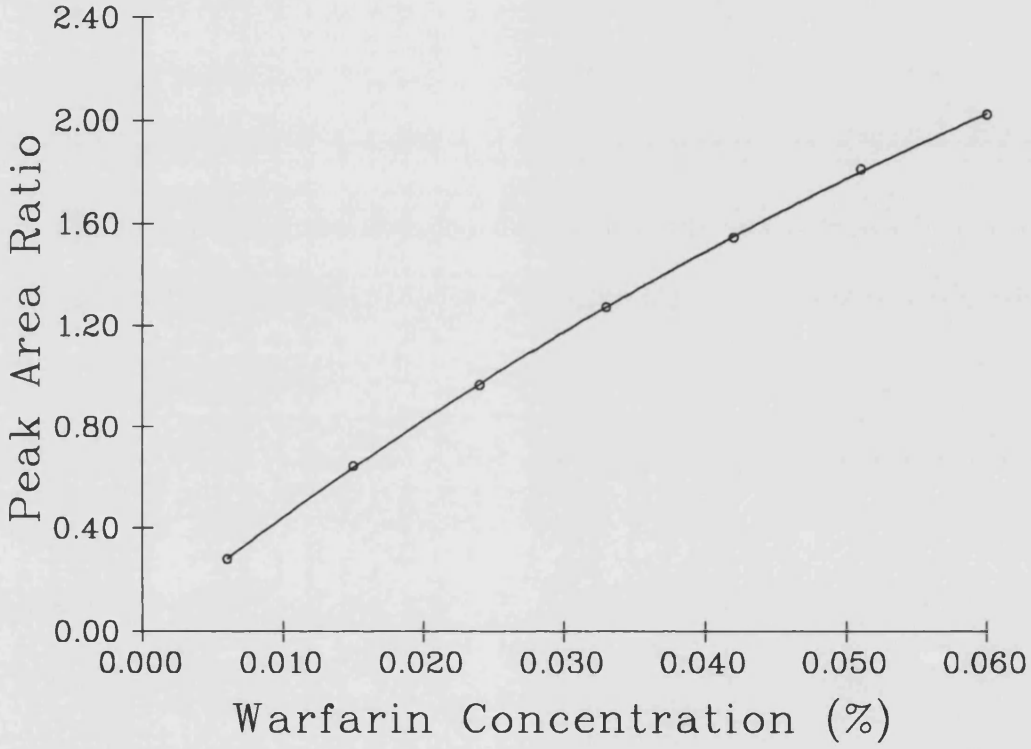


Figure 4.4 Peak area ratio *vs* warfarin concentration calibration graph (non-linear regression line shown)

STATISTICS

r-squared	0.99999	Correlation	0.99996

Equation of line:-

Peak Area Ratio = 0.02838 + (43.0273 × Warfarin Conc.) - (161.6696 × Warfarin Conc.^{2})

Although the data is useable, the better non-linear fit suggests that solubility is having an influence, or that the warfarin (free acid) is not partitioning fully into the ethyl acetate layer.

4.4.5 The reduction of warfarin by *Rhodococci* against time

72 hour cultures of ATCC 19070 (37°C in TSB) and ATCC 19140 (26°C in TSB) were prepared (incubation conditions as before).

Nine flasks were then prepared:-

1) TSB 40ml, ATCC 19070 72hr culture 5ml, 0.5% RS-warfarin 5ml

2) TSB 40ml, ATCC 19070 72hr culture 5ml, 0.5% S-warfarin 5ml

3) TSB 40ml, ATCC 19070 72hr culture 5ml, 0.5% R-warfarin 5ml

4) TSB 40ml, ATCC 19140 72hr culture 5ml, 0.5% RS-warfarin 5ml

5) TSB 40ml, ATCC 19140 72hr culture 5ml, 0.5% S-warfarin 5ml

6) TSB 40ml, ATCC 19140 72hr culture 5ml, 0.5% R-warfarin 5ml

7) TSB 40ml, ATCC 19070 72hr culture 5ml

8) TSB 40ml, ATCC 19140 72hr culture 5ml

9) TSB 45ml, 0.5% RS-warfarin 5ml

Samples were taken at 1hour, then periodically over the next 12 days

4.4.5.1 Results

Streak plates were produced from all flasks at the end of the experiment to check for any contamination. All flasks were shown to be clear.

Flasks 7 and 8 demonstrated that the *rhodococci* grew strongly in the media under the stated conditions.

Analysis of flask 9 gave a single warfarin peak throughout the experiment (ie. no chemical degradation products were seen). The warfarin concentration at the end of the experiment (288 hours) was not significantly different from the sample taken at one hour.

The amount of warfarin metabolism in flasks 1 to 6 was again disappointing when compared to that seen by Davis and Rizzo[77]. The levels were again too low to produce any significant reduction in the amount of remaining warfarin, but in the absence of warfarin alcohol standards (see section 3.5) the peak areas of the metabolites produced divided by the coumarin (internal standard) peak areas are shown below.

Flasks 1 to 3 ATCC 19070

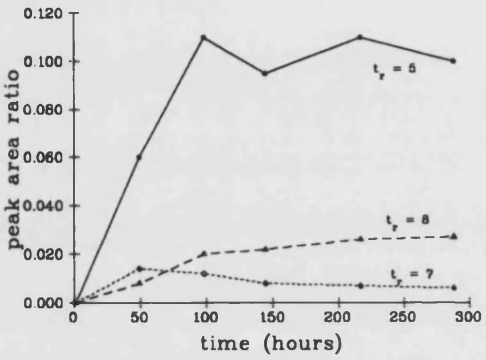


Figure 4.5 Flask one - RS-warfarin

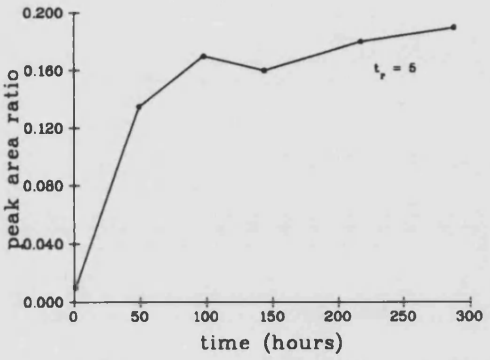


Figure 4.6 Flask two - S-warfarin

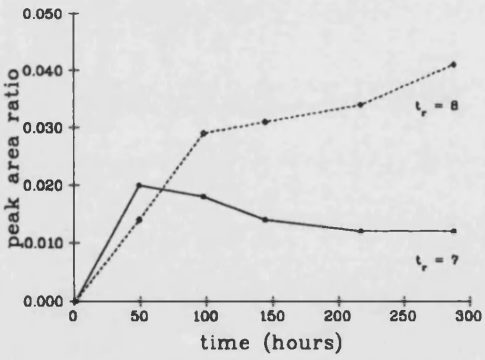


Figure 4.7 Flask three - R warfarin

Three additional peaks developed as the experiment progressed:-

1) t_r = 5 minutes corresponding to R–warfarin-R-alcohol or S–warfarin-S-alcohol

2) t_r = 7 minutes) corresponding to R-warfarin-S-alcohol or S-warfarin-R-alcohol

3) t_r = 8 minutes - identity unknown

(t_r =retention time)

From the graphs for flasks 2 and 3 it can be seen that the peak occurring at a t_r of 5 minutes has been produced from S-warfarin (no peak at this retention time if only R-warfarin is present) and therefore must be S-warfarin-S-Alcohol (Figure 4.8).

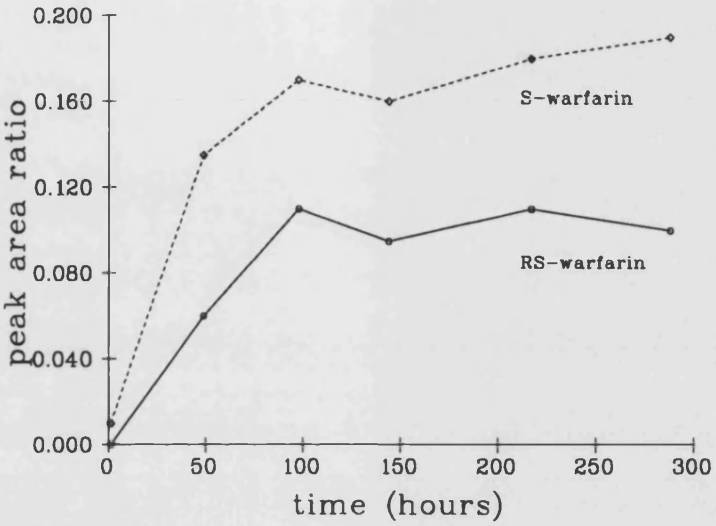


Figure 4.8 Production of S-warfarin-S-alcohol by ATCC 19070 when incubated with S-warfarin or RS-warfarin

By a similar argument, the peaks at 7 minutes and 8 minutes must be produced from R-warfarin. The peak at t_r = 7 must be R-warfarin-S-alcohol. It is interesting that in both flask 1 and flask 3, the quantity of this metabolite appeared to decrease after its initial increase (Figure 4.9).

These result show that ATCC 19070 preferentially metabolises S-warfarin (peak area ratios for R-warfarin reduction are a factor of ten lower), and produces the S configuration alcohol exclusively.

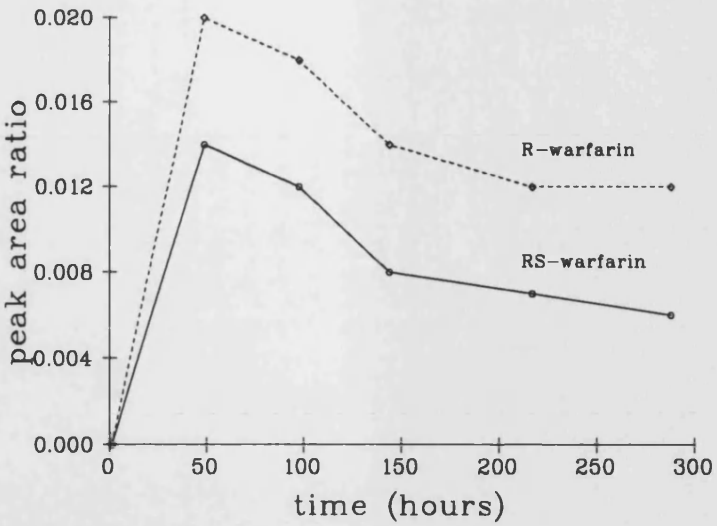


Figure 4.9 Production of R-warfarin-S-alcohol by ATCC 19070 when incubated with R-warfarin or RS-warfarin

Flasks 4 to 6 ATCC 19140

ATCC 19140 showed no significant reduction of R-Warfarin (flask 6). S-warfarin was reduced entirely to the S-alcohol (t_r = 5 min) as shown in Figure 4.10

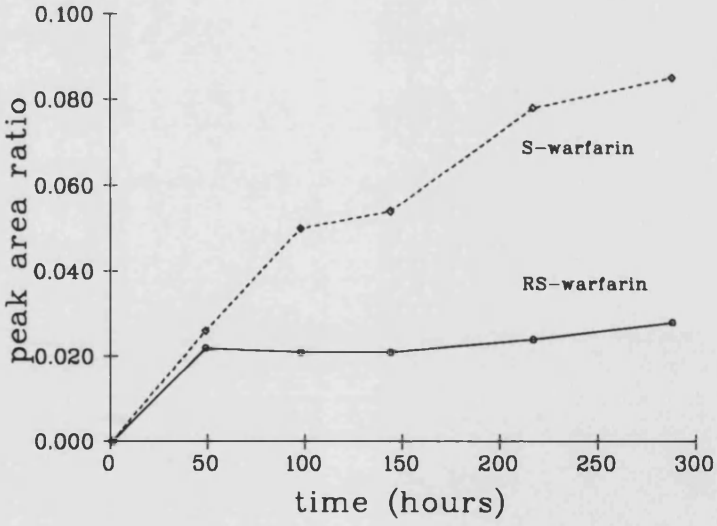


Figure 4.10 Production of S-warfarin-S-alcohol by ATCC 19140 when incubated with S-warfarin or RS-warfarin

4.5 The growth of *Rhodococci* in a fermenter

In spite of the relatively low percentage conversion of warfarin to its alcohols shown in the previous section, it was decided to attempt to grow both microorganisms in a fermenter. The high cell masses generated by such growth could then be used to see whether the percentage conversion could be increased, and whether it might be possible to extract the enzymes responsible.

4.5.1 Method

A 24 hour culture of either ATCC 19070 or ATCC 19140 was prepared in shake flask (conditions as before) containing 250ml of pre-sterilised TSB (37°C or 26°C respectively)

A 6 litre capacity fermenter (Figure 4.11) was filled with 4750ml of TSB and steam sterilised by autoclaving at 121°C, 15 p.s.i. for 20 minutes. Once the solution had cooled, the various connections to the fermenter vessel were made using standard aseptic techniques. These included the acid (1M orthophosphoric acid) and base (1M Sodium hydroxide) solutions which the fermenter adds to the vessel (automatically using peristaltic pumps) as required to maintain the pH, and the silicon based anti-foam solution. The appropriate temperature was set and the solution allowed to equilibrate.

The dissolved oxygen sensor was calibrated according to the manufacturers instructions by:-

a) sparging the solution with nitrogen with the impeller speed set to 700 r.p.m. Once the reading from the probe had settled, this was defined on the fermenter control panel as being a solution with a dissolved oxygen tension of zero.

b) sparging the solution with air at a flow rate into the vessel of 3 litres.min^{-1} with the impeller rate set at 400 r.p.m. This solution was taken to have a dissolved oxygen tension of 100%

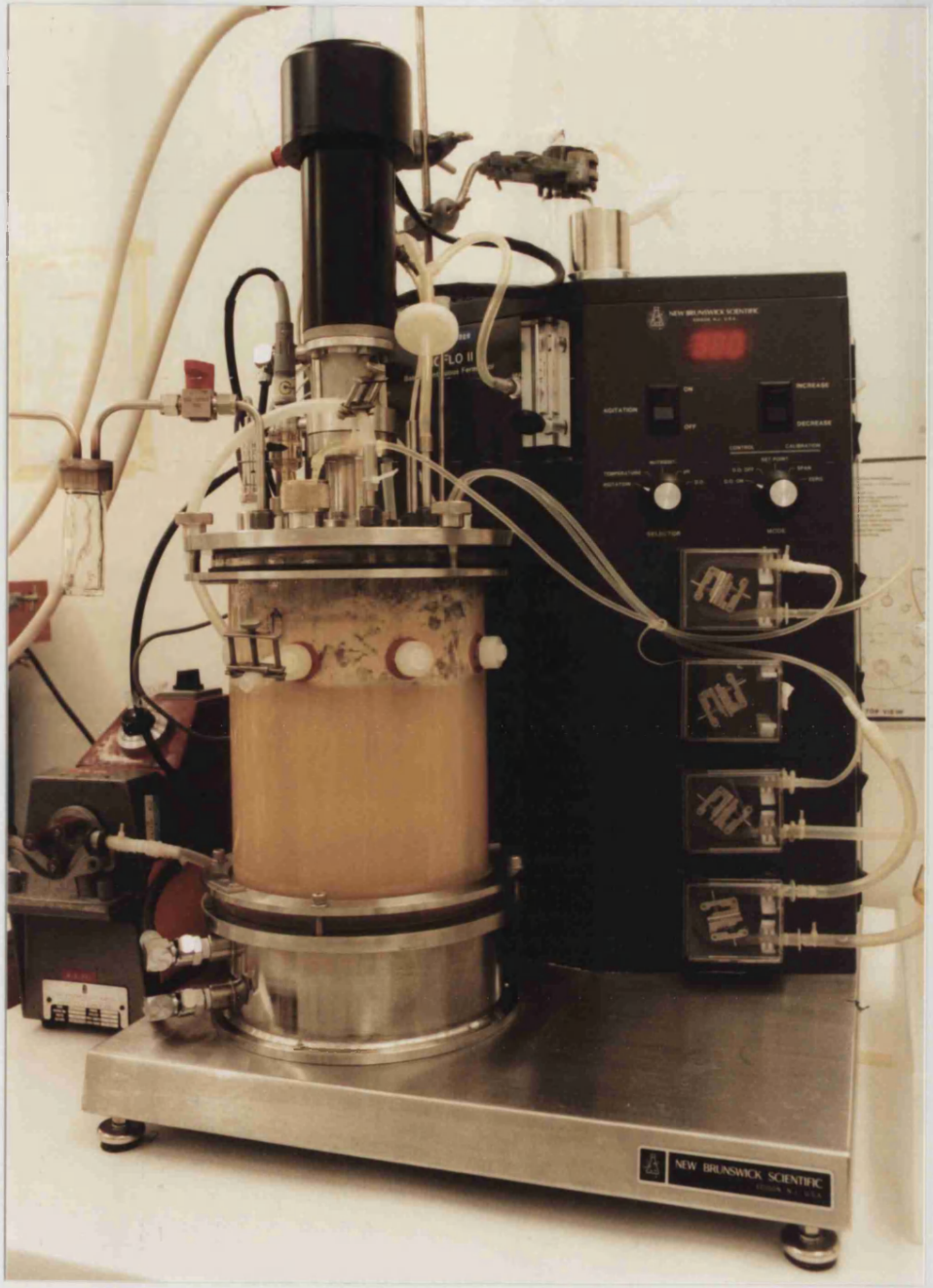


Figure 4.11 The Bioflow II fermenter

The air input flow rate was adjusted to 0.5 $l.min^{-1}$ and the fermenter was set to automatically maintain (by varying the agitation speed) a dissolved oxygen tension of 50%. Initially, before the addition of any culture, the dissolved oxygen tension was around 80% with the impeller operating at its minimum speed (around 60 r.p.m.)

The fermenter was set to maintain the culture pH at 7.2.

Once the 250ml of inoculum had been added, growth was allowed to proceed until the fermenter was unable to maintain the 50% oxygen tension with the impeller operating at 600 r.p.m. (42-48 hours)

Culture samples were taken periodically during the fermenter cycle and streak plates performed to check for contamination.

Cells were harvested by centrifugation at 2000 r.p.m. for 15 minutes in a 6 × 1 litre capacity centrifuge (School of Biochemistry). The supernatant was discarded then the cells were re-suspended in approximately twice the packed cell volume of a pH 7.4 tris(hydroxy-methyl)aminomethane (Tris) buffer. This served to wash the cells in order to reduce the concentration of any of the anti-foaming agent added during the fermentation.

Cells were re-harvested by centrifugation and this washing process repeated three times

The following wet cell masses of *Rhodococci* were obtained.

ATCC 19070 196g

ATCC 19140 134g

The cells were re-suspended at a concentration of 50% w/v in Tris buffer and stored at 4°C.

4.6 Metabolism of warfarin by the fermenter cell suspensions

A quick initial experiment with the cell suspension from ATCC 19070 showed encouraging results. 0.5ml of 0.5% S-warfarin solution was added to 4.5ml of the suspension. This was incubated in the incubator/shaker at a temperature of 37°C

and samples taken periodically for extraction according to the method outlined above. The results are given in Figure 4.12.

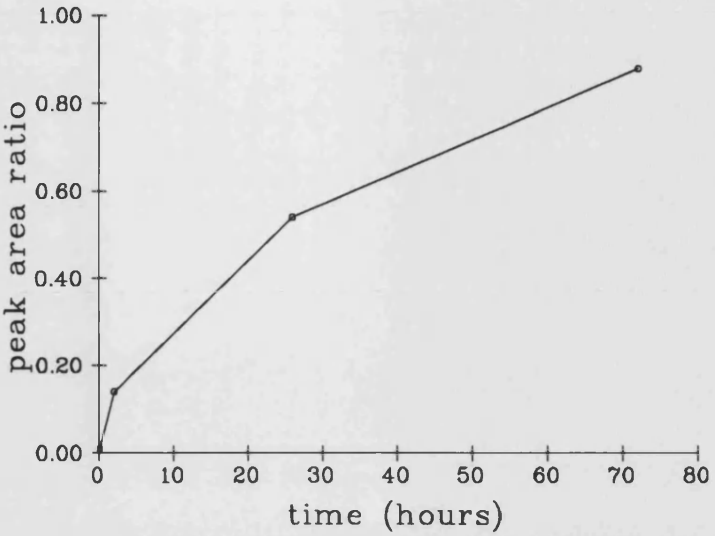


Figure 4.12 The production of S-warfarin-S-alcohol by a 50% w/v suspension of ATCC 19070 cells

Although this information is from a single flask, this system showed significantly more conversion than any of the previous experiments. This suggests that the degree of conversion is related to the cell mass present and that cells do not need to be actively growing in order for reduction of the aliphatic ketone group to occur.

5 NICOTINAMIDE ADENINE DINUCLEOTIDE

5.1 Introduction

Glucose Oxidase (considered earlier) is an Oxidoreductase which has a prosthetic group based on the flavin moiety. There are in excess of 550 commercially available oxidoreductases. Approximately 250 of these use coenzymes which contain the nicotinamide moiety, namely nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). The reduced forms of these two coenzymes are referred to as NADH and NADPH respectively.

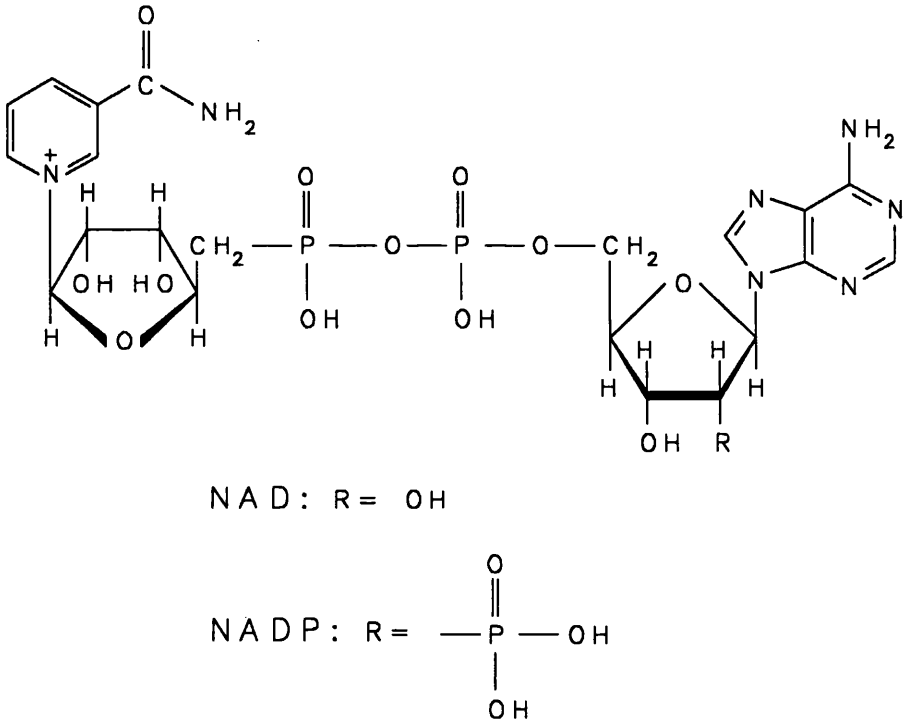


Figure 5.1 Structures of the coenzymes NAD^+ and $NADP^+$

PLEASE NOTE: Where information in the text refers to both NAD^+ and $NADP^+$, this will be written as $NAD(P)^+$. Similarly, the reduced cofactors will be collectively referred to as NAD(P)H.

Nicotinamide is the functional part of the coenzyme molecule in reduction/oxidation reactions.

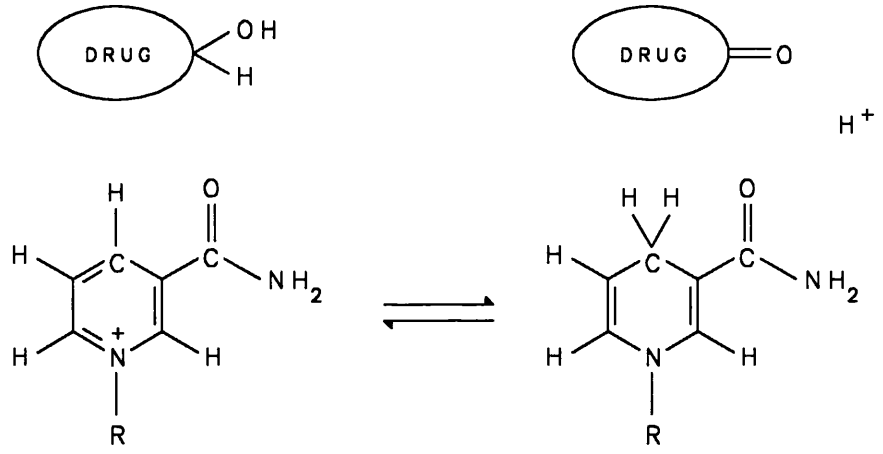


Figure 5.2 The oxidised and reduced forms of the nicotinamide moiety (NAD or NADP coenzyme)

The development of a biosensor based on an $NAD(P)^+$ dependant oxidoreductase requires additional problems to be overcome when compared to existing biosensor technologies (for example glucose biosensors)

1) NAD^+ and $NADP^+$ are cofactors (rather than prosthetic groups) and are able to dissociate from the enzyme. It is therefore necessary to immobilise them at the electrode surface (as well as the enzyme), in order that sensor output does not decrease with time. Additionally, this immobilisation needs to be performed in such a way that enzyme activity is not dramatically affected. ie. any chemical modification made to the cofactor should not stericaly hinder its ability to access the active site on the enzyme.

It should be noted though that it may be possible to modify the cofactor in order to allow it to have a secondary function as an electron transfer mediator between the active site of the enzyme and the electrode surface.

2) If the enzyme is acting to oxidise a substrate the $NAD(P)^+$ will consequently end up in its reduced form NAD(P)H. In order to produce a biosensor the cofactor would need to be re-oxidised at an anode.

$$NAD(P)H \xrightarrow{\text{Anode}} NAD^+(P) + 2H^+ + 2e^-$$

Unfortunately, although this reaction is feasible at an electrode, it requires a large positive potential (eg. + 1V *vs* AgCl at a clean platinum electrode). However, several novel "conducting organic salt" electrode materials allow this reaction to proceed at low anodic potentials.

e.g. The oxidation of ethanol by alcohol dehydrogenase (ADH)

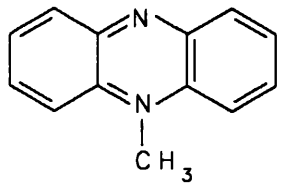
$$CH_3CH_2OH + NAD^+ \xrightarrow{\text{ADH}} CH_3CHO + NADH + H^+$$

$$NADH \longrightarrow NAD^+ + 2H^+ + 2e^-$$

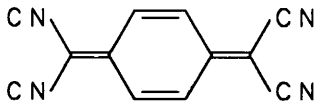
$$\downarrow$$

ANODIC CURRENT

This reaction can occur at 0.0V (with respect to a Ag/AgCl reference electrode) at a PVC bound NMP^+TCNQ^- electrode[101] (Figure 5.3)



NMP



TCNQ

Figure 5.3 Structure of N-methylphenazinium (NMP) and Tetracyanoquinodimethane (TCNQ)

The mechanism by which this occurs at such surfaces is not well understood. Some dissolution of the electrode surface may be occurring so that an electron transfer mediator is formed in situ.

3) If the $NAD(P)^+$ dependent enzyme is performing a reduction the $NAD(P)^+$ will end up in its oxidised form. This oxidised coenzyme will need to be reduced (either directly or indirectly) at the electrode surface in order to regenerate the active cofactor. However, this does not happen (Figure 5.4). Although a small amount of the active NAD(P)H is regenerated, a series of dimers and other reduction products are formed[102,103,104].

Analysis of what happens when the cathodic reduction of $NAD(P)^+$ is attempted is the subject of this chapter.

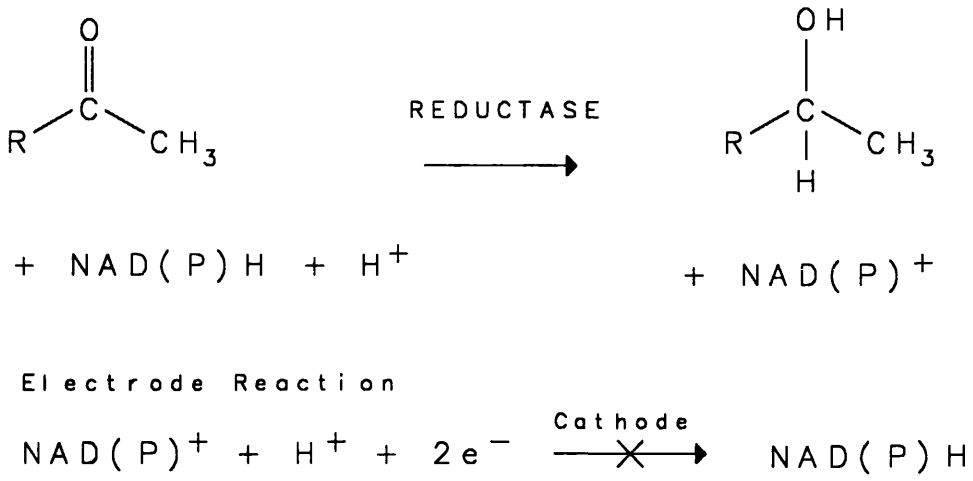


Figure 5.4 Desired sequence of events at biosensor based on a NAD(P)H dependent reductase

5.1.1 The Regeneration of NAD(P)H from NAD(P)$^+$

If a method for the regeneration of 100% active NAD(P)H without using an enzyme were to be found, it would be useful in a variety of different scientific areas. For example, during the synthesis of compounds using biochemical reactors[105] it is often necessary to add a second substrate (to force the equilibrium in the desired direction a large excess may be required) and possibly a second enzyme, in order to regenerate the active cofactor for use in the desired enzyme reaction (Figure 5.5). This incurs the additional difficulty of having to remove unwanted products and reactants from the mixture.

From the point of view of reagentless biosensor development, there is a need to find an electrochemical system by which active NAD(P)H can be regenerated at low negative potentials without producing inactive products. Various organometallic compounds of Rhodium are considered later as possible reaction-modifying mediators.

Much effort has been put into the identification of the various products from the reduction (either chemical or electrochemical) of NAD(P)$^+$ [102,103,104]. Experiments which identify the exact identity of inactive products are less important at this stage than

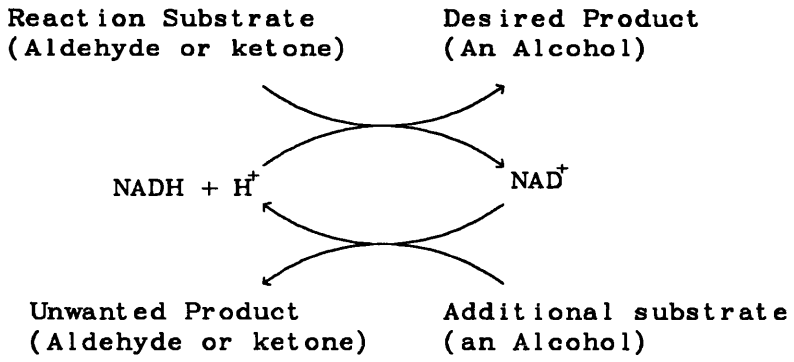


Figure 5.5 The regeneration of a cofactor in a biochemical reactor

experiments which can demonstrate a change in the profile of the products formed when a particular mediator is present.

5.2 HPLC Analysis of NAD^+ and its Reduction Products

A reversed phase gradient HPLC system was used to separate NAD^+ from its reduction products and is detailed below.

5.2.1 Equipment

LDC Spectromonitor III variable wavelength UV detector (TWO) or LDC Spectromonitor 5000 photodiode array detector (capable of recording at more than one wavelength at the same time)

LDC Constametric III dual piston metering HPLC pumps (TWO)

LDC GM4000 gradient programmer

BBC Servogor SE120 chart recorder (TWO)

HPLC column water bath - Grant SU6 re-circulating water bath heater running against a Haake EK12 water bath cooling coil.

Remote immersible magnetic stirrer (Rank Bros., Bottisham, Cambridge)

Rotary evaporator, Büchi rotavapor

Wenking model LB75M potentiostat (UK distributor: Linton Instrumentation, Harlow, Essex, England) The external reference voltage source for the potentiostat was from a Metrohm 612 VA Scanner (Metrohm Ltd., Herisau, Switzerland) used in its fixed voltage mode.

Thandor TM356 Digital Multimeter (RS components LTD, Corby, Northants, England)

Mettler AE163 4 or 5 decimal place analytical balance

5.2.2 Materials

5.2.2.1 Chemicals

Ammonium bicarbonate (99%), sodium pyrophosphate decahydrate (A.C.S. Reagent) were obtained from the Aldrich Chemical Company Ltd., Gillingham, Dorset, England.

Acetone and Methanol (HPLC grade) were obtained from Fisons Scientific, Loughborough, Leicestershire, England.

Mercury (triple distilled) was obtained from Belgrade Mercury Ltd, Belgrade, England. Any surface film was removed from the mercury allowing it to fall through a small pin-hole in a piece of filter paper twice immediately before use.

5.2.2.2 Nicotinamide Co-factors

The following were obtained from the Sigma Chemical Company Ltd., Poole, Dorset, England.

β-Nicotinamide adenine dinucleotide (grade III-C, product No. N-1511)

β-Nicotinamide adenine dinucleotide (grade III, product No. 260-101) 1mg (1.46 micromole) pre-weighed vials

β-Nicotinamide adenine dinucleotide, disodium salt, reduced form, (Grade III, product No. 340-101) 1mg (1.28 micromole) pre-weighed vials.

5.2.2.3 HPLC Columns

Analysis column: LKB (Pharmacia) Ultrapack 5K ODS-120T column (5µm particle size ODS, 4.6mm internal diameter, 250mm length)

Mixing Column: An old Chromotech Hypersil column (5µm particle size ODS, 5mm internal diameter, 150mm length) scavenger/mixer column was used between the two pumps and the injection loop. The purpose of this was to ensure adequate solution mixing (high pressure) and to try protect the analysis column against the relatively low pH of the mobile phase.

5.2.2.4 Other Items

0.2µm 25mm diameter cellulose nitrate membrane filters and 0.45µm 47mm diameter cellulose acetate membrane filters were obtained from Whatman Ltd, Maidstone, Kent, England.

5.2.3 Preparation of solutions for HPLC

5.2.3.1 NAD and NADH standard solutions

These solutions were prepared immediately before use from commercially available 1mg pre-weighed vials. For the isocratic test runs the cofactor was dissolved in mobile phase. For gradient HPLC, samples were reconstituted with a solution equivalent to the starting composition of the mobile phase. This solution composition is referred to as "Initial Mobile Phase" later in the text.

Two types of calibration method occurred:-

1) To establish the linearity of the assay, a set of seven solutions were prepared containing both NAD^+ and NADH in concentrations ranging from 0.005mM to 0.1mM of each compound. These were then put on to the column in a random order.

2) For individual analysis of a reaction mixture, a solution containing a high concentration of each compound (0.1mM) and a solution containing a low concentration of each compound (0.02mM) were analysed before and after the reaction mixture. The concentrations of NAD^+ and NADH in the reaction mixture were then calculated by extrapolation.

5.2.3.2 Electrolysis Buffer Solution

The buffer solution used for the electrochemical reduction experiments consisted of dipotassium hydrogen phosphate 0.1M and tetraethylammonium chloride 0.05M in RO water, adjusted to pH 7.00 using 5M and 0.5M hydrochloric acid in RO water. This solution was deoxygenated by bubbling oxygen free nitrogen through it (via a glass sinter) for 15 minutes prior to use.

The purpose of the tetraethylammonium chloride is for this salt to preferentially adsorb at the mercury surface and therefore reduce the adsorption of NAD^+.

5.2.3.3 NAD^+ Stock Solution

In order to semi-quantitatively match the chemical reduction experiment to the electrochemical reduction experiments, a stock solution of 2mM NAD^+ in the electrolysis buffer described earlier was prepared. The same solution was then used for all three reductions (chemical and electrochemical at -1.1V and -1.8V) on the same day.

5.2.3.3.1 Beer-Lambert plot for NAD^+

In order to assay the starting concentration of NAD^+ in the stock solution a Beer-Lambert plot was constructed. Quantities of NAD^+ were accurately weighed directly into 50.0ml class A volumetric flasks and made up to volume with electrolysis buffer. A 5.00ml sample of each solution was then diluted to 50.0ml with "initial mobile phase". This 1 in 10 dilution mimicked the dilution which occurred when electrolysis samples were diluted for HPLC analysis. A 10%v/v solution of electrolysis buffer in initial mobile phase was used

as a blank. Absorbances were measured using a Perkin Elmer λ3 dual beam spectrophotometer using matched quartz cells with a 1.0cm path length.

Weight of NAD^+	Concentration in solution	Absorbance at 260nm
19.8mg	55.9 µM	0.989
15.6mg	44.0 µM	0.766
11.8mg	33.3 µM	0.591
7.9mg	22.3 µM	0.397
3.8mg	10.7 µM	0.197

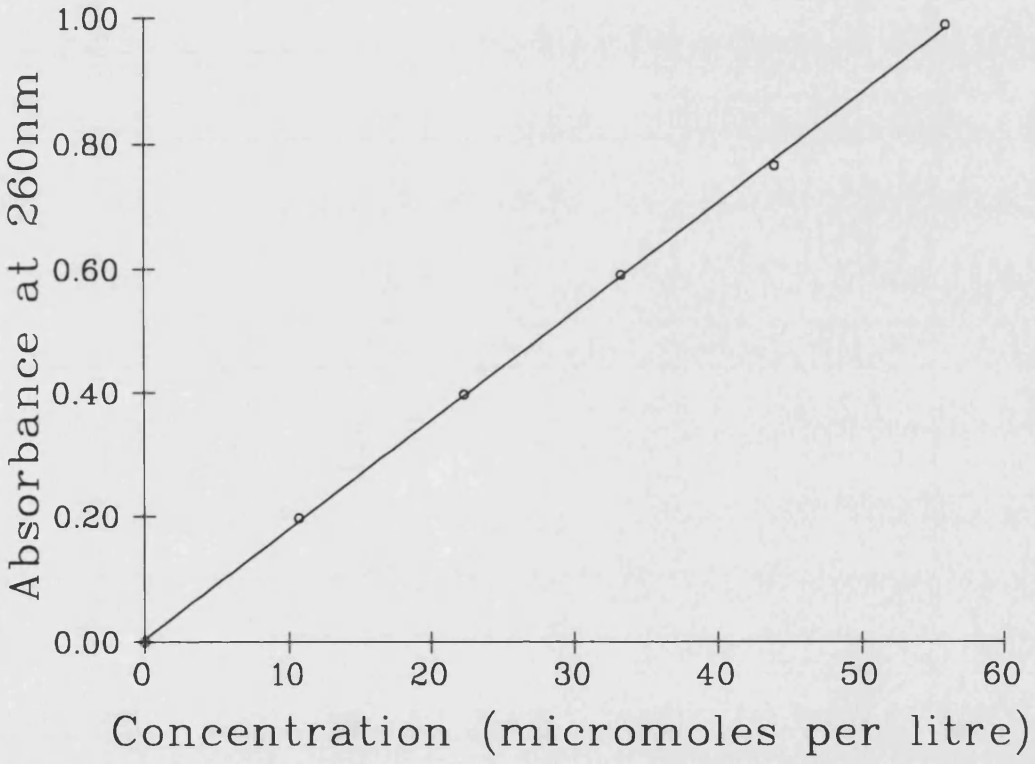


Figure 5.6 Beer-Lambert plot for NAD^+ at 260nm

STATISTICS

r-squared	0.99992	Correlation	0.99987
Calculated intercept	0.0042	Standard deviation	0.0047
Calculated slope	0.01754	Standard deviation	0.00014

Using the equation A = E.c.l, where A = absorbance, c = concentration and l = path length in cm.

Calculated extinction coefficient E_{260}^{mM} = 17.54 (pH 8.2)

The manufacturers quote a value of 17.7 (pH 7.0) [106]

5.2.3.4 Electrochemically reduced NAD^+

According to Jaegfeld[103] the reduction of nicotinamide adenine dinucleotide proceeds in two steps. A one electron step at -0.9V vs s.c.e. an a mercury electrode which produces a radical which can dimerise to give enzymatically inactive products. A second step at -1.7V vs s.c.e which produces compounds with some enzymatic activity.

For the purpose of this study, reductions of NAD^+ were carried out at -1.1V and at -1.8V vs s.c.e. electrode in the electrochemical cell shown below (Figure 5.7).

The air-tight lid of the cell had two additional ports not shown in Figure 5.7. One of these had a dip tube fitted to allow de-oxygenation of the solution by bubbling with pre-humidified nitrogen. During the experiment the nitrogen was re-directed to flow over the surface of the reaction solution at a flow rate which produced a small depression in the liquid surface.

The electrochemical cell and all associated glassware was cleaned after each experiment by immersing in a chromic acid solution for 30 minutes followed by rinsing with copious amounts of distilled water. A final rinse of RO water was used prior to drying in a hot air oven.

3.0ml of mercury was used in the cell and this had a physical electrode surface area of approximately $9cm^2$. The salt bridge consisted of a piece of glass tubing into which a 7mm

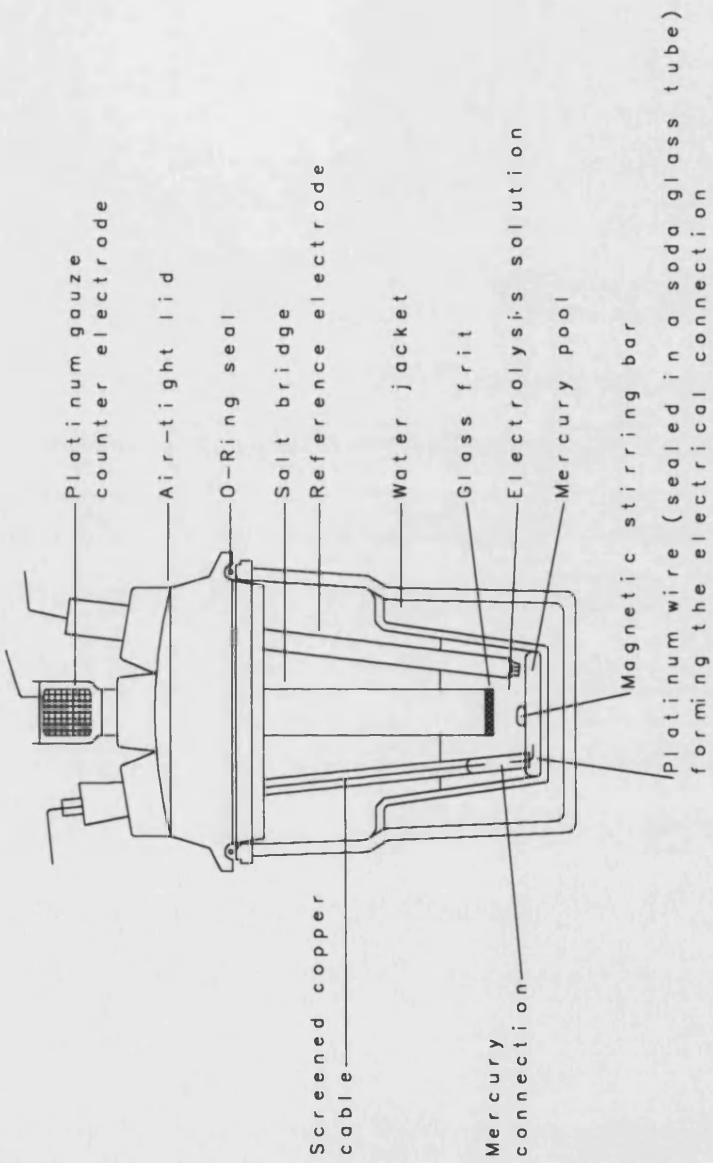


Figure 5.7 Cell configuration used for the electrochemical reduction of NAD^+

diameter No. 5 glass frit had been sealed. The tube was filled with a solution consisting of 3M potassium chloride in RO water which had been solidified by the addition of purified agar at a concentration of 1%$^{w}/_{v}$. A space was left unfilled in the top of the tube to accommodate the counter electrode. This was fabricated from a 2cm x 10cm piece of platinum gauze, which was rolled up and immersed in a 3M solution of potassium chloride in RO water. A radiometer K401 s.c.e. reference electrode was positioned just above the mercury surface. Electrical connection to the mercury pool was achieved using a piece of 0.5mm diameter platinum wire which had been sealed into glass. This was then dipped in to the mercury pool.

Electrochemical reductions were all carried out at 10°C. The teflon coated magnetic stirring rod (3mm diameter x 10mm) was used to keep the solution well mixed and to help renew the mercury surface. It was rotated as quickly as possible in order to create standing waves on the mercury surface without causing the surface to separate into droplets.

5.2.3.4.1 Electrochemical Reduction Methodology

8.00cm^3 of pre-cooled electrolysis buffer was carefully added to the cell avoiding getting any of the solution on the sides of the cell above the liquid level. The appropriate electrode potential was applied and the current allowed to settle until stable. The working electrode potentials were checked using a high input impedance calibrated multimeter and were found to be -1.101V and -1.800V respectively. The cell was disconnected from the potentiostat and 8.00cm^3 of a 2mM solution of NAD^+ (pre-cooled) was then added to the cell. After a 10 minute period to allow for mixing and temperature equilibration the cell was re-connected and the reduction allowed to proceed. During the -1.8V reduction a significant amount of gas evolution (presumably hydrogen) occurred at the mercury surface. When the current flowing had dropped to the same order of magnitude as the background current a 1.00cm^3 sample was taken and diluted to 10.0cm^3 with "initial mobile phase", and the sample analyzed immediately using the gradient HPLC system.

5.2.3.5 Sodium Borohydride reduced NAD

5.2.3.5.1 Method One

The first time the reduction was tried 150mg (0.21 millimole) of NAD^+ (as free acid) was reacted with 10mg (0.26 millimole) of sodium borohydride. Assuming the production of approximately 3 moles of hydrogen from each mole of sodium borohydride, there was an almost four times molar excess present. The compounds were dissolved in 5.0ml of RO water and allowed to react at room temperature.

Both NAD and NADH absorb at 259nm. Another absorption maxima is seen at 340nm for NADH. The reaction was monitored by taking a 40microlitre sample and diluting to 10ml with further RO water (1 in 250). The absorbance was then measured at 340nm. Based on total conversion to NADH and on the manufacturers quoted extinction coefficient $E_{340}^{mM} = 6.2$, a diluted sample should have had an absorbance of approximately 1 when the reaction is complete. When this reaction was performed, a sharp rise in absorbance occurred (absorbance at t=0 was 0.007) followed by gradual decline (Figure 5.8). This served to illustrate the care needed when preparing such samples. NADH is unstable in acidic solutions[107] and as well as forming various breakdown products it is likely that dissolved oxygen may have re-oxidised any NADH formed. Additionally, an un-buffered solution of NAD^+ is acidic and will tend to decompose the sodium borohydride as it is added. Visually, when the sodium borohydride was added gas was evolved and the solution became a pale yellow colour which then faded over the next 30 minutes. This colouration has been observed by other workers[108]

5.2.3.5.2 Method 2

The experiment was repeated in 5ml of 0.1M sodium pyrophosphate solution in RO water which had been adjusted to pH 9.0 using a combination of 5.55M and 1M solutions of Hydrochloric acid in RO water. The solution was purged for 10 minutes with oxygen free nitrogen prior to the addition of the NAD^+. Before the addition of the sodium borohydride the absorbance was 0.008. The absorbance increased to a value of around 0.7 within one

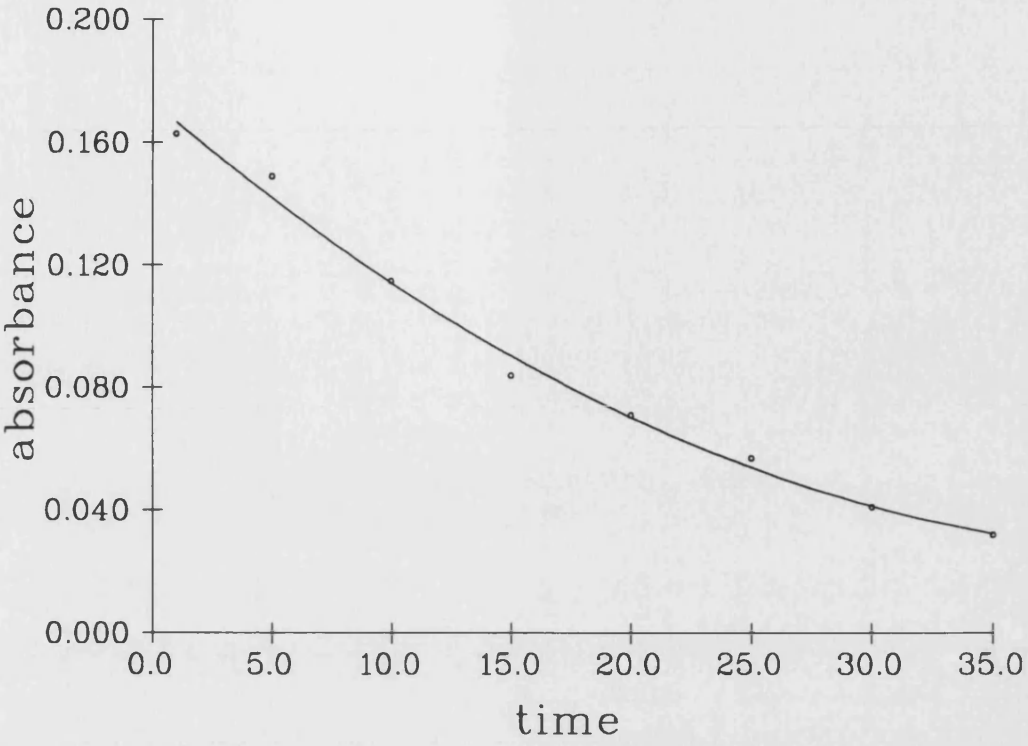


Figure 5.8 The absorbance of the reaction mixture against time for sodium borohydride reduction of NAD in water (unbuffered)

minute and remained at this value (Figure 5.9). 3ml of HPLC grade acetone was then added to react with any excess sodium borohydride. Oxygen free nitrogen was used to displace any air from above the reaction mixture and the flask was sealed using a balloon arrangement to allow for the expansion of any gas produced. 12 hours later the solution was evaporated to dryness in a rotary evaporator. The residue was dissolved in a small volume of RO water, which had been purged with nitrogen, and filtered through a 0.2µm membrane filter into a 20ml class A volumetric flask. The filter was washed with several aliquots of RO water and the flask was finally made up to volume and stored in a refrigerator. This solution (produced from the equivalent of a 10mM solution of NAD^+ once sampling and dilution had been taken into account) proved to be very useful for the development of HPLC methodology. For injection onto the column it was diluted 1 in 100 with initial mobile phase. The sizes of individual peaks from this solution varied however as the solution aged. To get a more representative idea of the products of the sodium

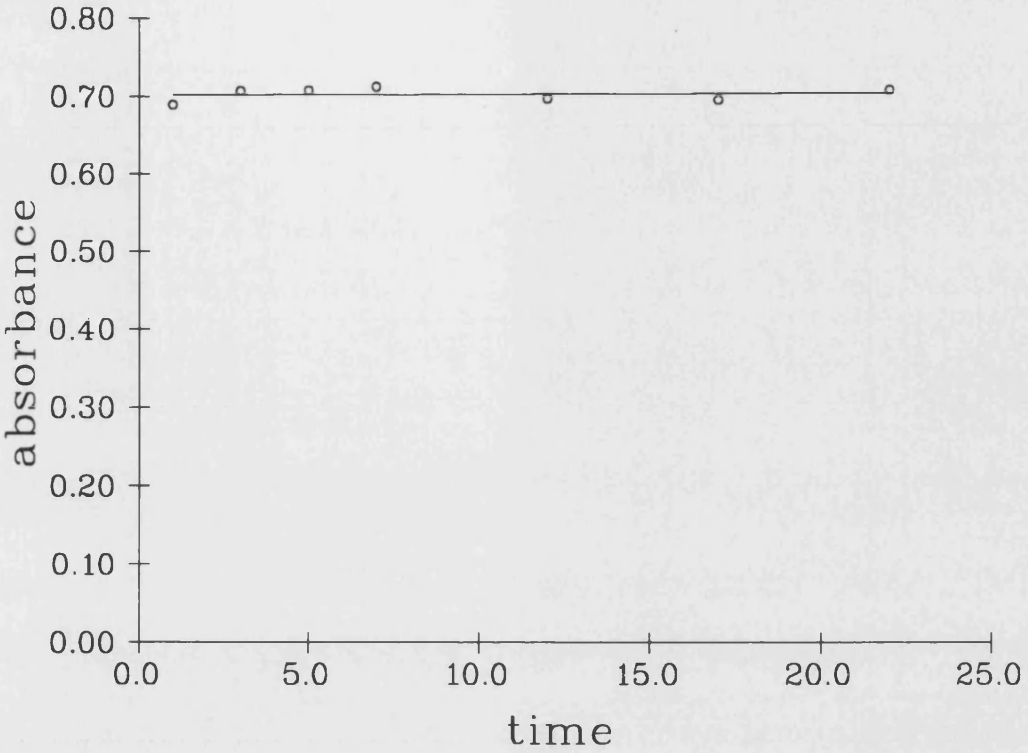


Figure 5.9 The absorbance of the reaction mixture against time for sodium borohydride reduction of NAD in water (buffered, pH 9.0)

borohydride reduction of NAD^+ method 3 was used.

5.2.3.5.3 Method Three

For this third borohydride reduction method $5cm^3$ of a 2mM stock solution of NAD^+ in electrolysis buffer (see section 5.2.3.3) was diluted with a further $5cm^3$ of electrolysis buffer. Oxygen free nitrogen was passed through the solution for 15 minutes prior to the addition of a large excess of sodium borohydride (15mg). The solution was allowed to react (with stirring) until hydrogen evolution subsided. $1.00cm^3$ of this solution was then diluted to $10.0cm^3$ with initial mobile phase and analyzed immediately using HPLC.

5.2.4 HPLC Methodology

5.2.4.1 Methodology Development

Three different HPLC mobile phase/gradient combinations were tried. The gradients were achieved using two LDC Constametric III dual piston metering HPLC pumps controlled by a LDC GM 4000 gradient programmer (high pressure mixing). The output from the two pumps was combined using a T-piece connector then passed through a 10cm x 5mm internal diameter column containing 1mm diameter glass beads to ensure adequate mixing. Subsequently, this column was replaced with an old analytical column (5µm particle size ODS, 5mm internal diameter, 150mm length) in order to achieve good mixing and to try and protect the analytical column from the relatively high pH. There was a minimum of fine bore tubing between the sample injection loop and the analytical column.

Jaegfeldt[103] used a mobile phase consisting of 30% water, 1-6% ethanol and 0.1M ammonium bicarbonate to 100%. Each run was "started isocratically at 1% ethanol and continued with a linear segment of increasing ethanol concentration up to about 4% ethanol". Following elution of the peaks the ethanol was further increased to 6% in order to wash the column. Timings were not given.

Umeda *et al*[109] used the starting composition from Jaegfeldts gradient system isocratically but with ethanol replaced by methanol (ie. 30% water, 1% methanol, 69% 0.1M ammonium bicarbonate).

Miksic and Brown[104] used a 60% solution of methanol in water varying from 2% to 30% with 0.02M potassium dihydrogen phosphate (pH 7.0) 98% to 70%. This gave a methanol concentration varying between 1.2% and 18%. A concave gradient program was used.

After many isocratic test runs the first gradient system (which used the same overall variation of alcohol concentration as Jaegfeldt[103]) was tried:-

GRADIENT 1

Mobile Phase A 30% water 70% 0.1M ammonium bicarbonate

Mobile Phase B 30% water 60% 0.1M ammonium bicarbonate 10% methanol

Time (minutes)	% mobile phase A	% mobile phase B	% methanol
0	90	10	1
2.5	90	10	1
7.5	60	40	4
15	60	40	4
20	40	60	6
25	40	60	6
30	90	10	1
35 (0 again)	90	10	1

Because of the large dead volume before the analytical column (due to the mixing column) there was a considerable delay (3 minutes with the glass bead [short] mixing column and 5 minutes using the old ODS [long] mixing column) before the set gradient occurred at the column (see 5.2.4.2).

When chromatograms obtained using gradient 1 were examined, the last peak from any analysis occurred as the methanol concentration increased towards 6%. Additionally, due to the 5 minute delay time once the long mixing column was in use, the initial isocratic section with 10% mobile phase B was un-necessary. Gradient 2 was devised to take these points into account.

GRADIENT 2

Mobile Phase A 30% water 70% 0.1M ammonium bicarbonate

Mobile Phase B 30% water 60% 0.1M ammonium bicarbonate 10% methanol

Time (minutes)%	% mobile phase A	% mobile phase B	Methanol
0	90	10	1
25	40	60	6
30	90	10	1
40 (0 again)	90	10	1

A gradient system based on the one used by Miksic and Brown[104] was also tried

GRADIENT 3

Mobile phase A 0.02M potassium dihydrogen phosphate adjusted to pH 7.0 with 1M sodium hydroxide

Mobile Phase B 60% methanol in water

Time (minutes)	% mobile phase A	% mobile phase B	% methanol
0	100	0	0
30	70	30	18
31	100	0	0
36 (0 again)	100	0	0

The program "EXP 2" was selected on the gradient programmer in order to give a concave increase in methanol concentration. This gradient worked less well than gradient 2 and was unable to provide full baseline separation of NAD^+ and NADH. In addition, compounds were eluting before the gradient had really started (once the mixing column delay had been accounted for). In hindsight this apparent poor performance may have been due to deterioration of the column. When gradient 2 was returned to the column performance deteriorated within the next 8 hours of use to the extent that it was relegated to the role of mixing column (see 5.2.4.3).

5.2.4.2 Mobile phase mixing efficiency and pump performance

Because of the use of high pressure mixing and because of the design of the HPLC pumps available for this work the pumps were likely to be inaccurate if asked to supply at very low flow rates. e.g. if pump A was set to provide 98% of the flow and pump B was set to provide only 2% of the flow it was likely that pump B would provide no output at all. To assess the performance of the pumps and the mobile phase mixing two short experiments were performed.

1) To assess the actual percentage of mobile phase provided by the pumps compared to the percentage requested by the gradient programmer.

Mobile phase A was pure water

Mobile phase B was 1% acetone in water

Absorbance was recorded at 300nm. With the analytical column in place (in order to maintain the back pressure) the gradient programmer was set to decrease from 100% mobile phase A down to 0% mobile phase A in 10% jumps every 3 minutes. The Actual percentage of mobile phase being provided by the pumps was then calculated. The flow rate was set at a total of $1.5cm^3min^{-1}$ and the solution was collected for each 3 minute period. This volume was not found to vary significantly from the 4.5ml expected.

Set % mobile phase A	Actual % provided by the pumps
20	18.75
30	28.57
40	38.39
50	48.21
60	58.93
70	67.86
80	78.57
90	89.29

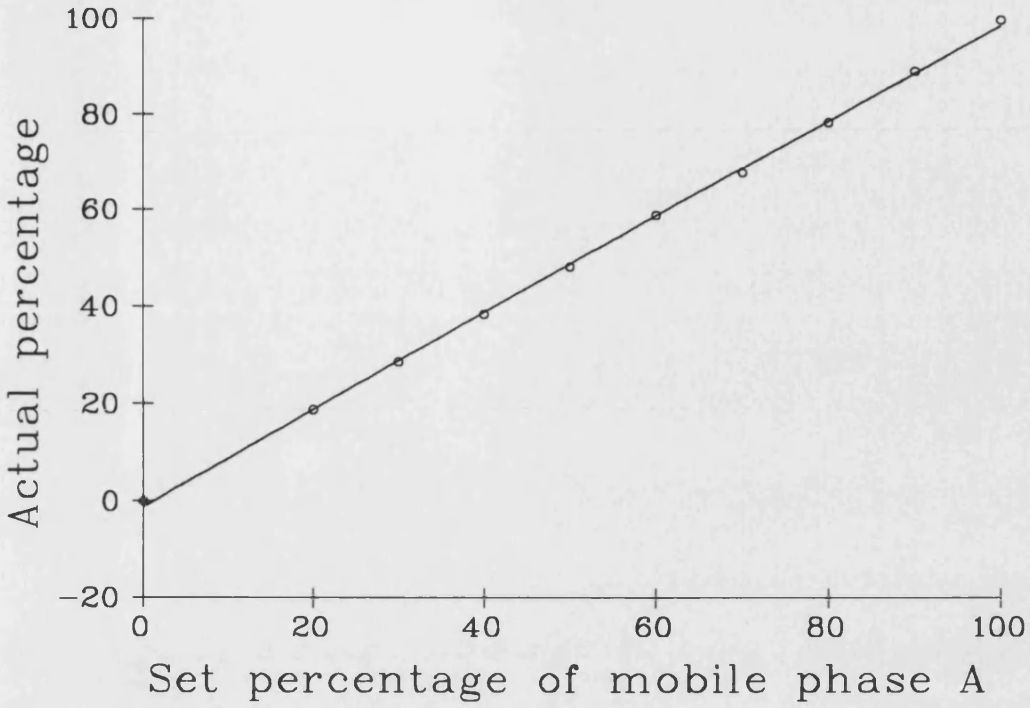


Figure 5.10 HPLC pump / mixing performance test

2) Gradient 1 was used to assess the performance of the short glass bead mixing column and the longer ODS filled guard/mixing column. The same mobile phase combination as above was used but this time the analytical column was removed and the UV detector placed where the column would have been. From this it was possible to see the difference in the delay time produced by the two columns.

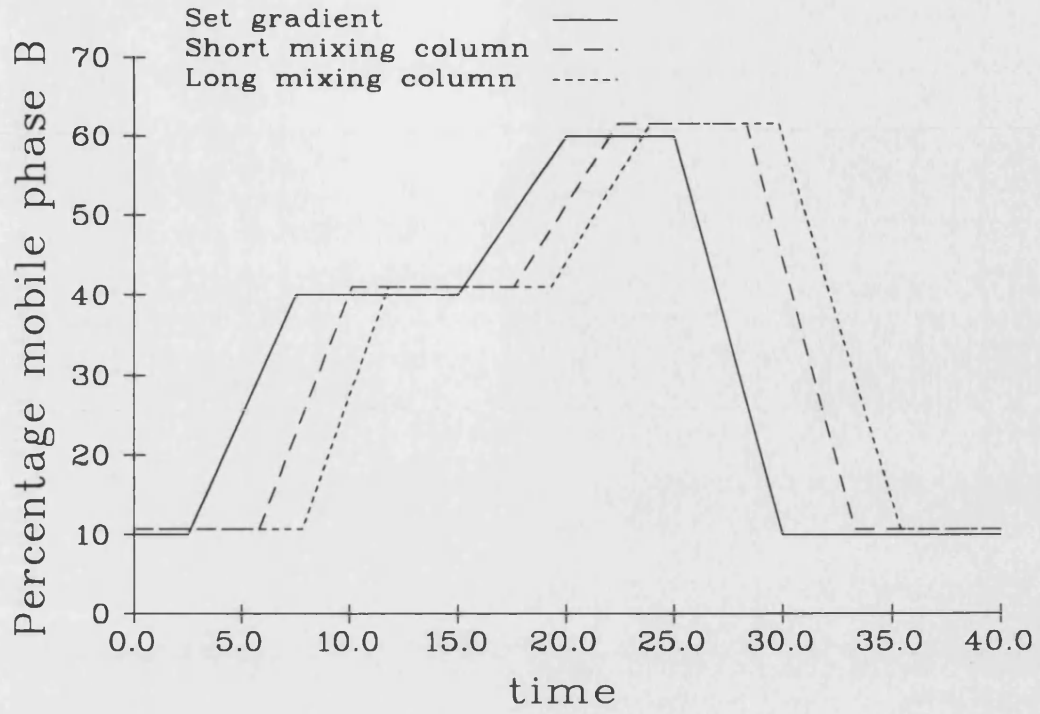


Figure 5.11 Mixing column performance showing the measured percentage of mobile phase B against the percentage set by the gradient controller

The results from this are shown in Figure 5.11. These results demonstrate adequate mixing and pump performance.

5.2.4.3 Analytical column life

On two occasions during this work the first analytical column used (Chromotech) started to give two peaks for each expected peak. On examination, the centre of the top of the column showed some erosion. The problem was cured by filling this depression with a slurry consisting of Lichosorb ODS powder mixed with a small amount of HPLC grade acetone and allowing it to dry. Once sufficient material had been added the column end cap was refitted.

Eventually, the column failed to fully resolve NAD^+ and NADH and was replaced with the longer LKB Ultrapack column. The old chromotech column was then used as a mixing/guard column. It was hoped that this would help to protect the new column from

being damaged by the high pH. When this was done the back pressure increased from approximately 2000 p.s.i. to nearly 3000 p.s.i. As can be seen from Figure 5.11 pump performance and mobile phase mixing were not adversely affected by this pressure increase.

The new column gave longer retention times due to its increased length. However the peaks produced were much sharper. Time limitations prevented the re-running of all samples on this column although this would have been desirable. Column details and gradients used are included in the results section.

5.2.4.4 Standard HPLC Conditions

The mixing/guard column was at room temperature.

The analytical column was held at 30°C by immersing it in a controlled temperature water bath.

The mobile phase flow rate was $1.5cm^3min^{-1}$

The Sample injection loop had a volume of 50µL.

Absorbance was recorded at both 260 and 340nm.

5.2.5 Results and discussion

5.2.5.1 Assay Calibration

The results below are from the LKB Ultrapak column using gradient 2 and the long mixing column. The peak areas are those measured at 260nm.

	Integrator peak area (260nm)	
Concentration of Solution (mM)	NAD^+	NADH
0.005	280750	239390
0.01	585720	484730
0.02	1209600	963960
0.04	2374200	1872500
0.06	3578500	2970000
0.08	4835400	3719600
0.1	6076900	4629000
r-squared	0.99995	0.99999
correlation	0.99994	0.99998
calculated intercept	-29820	24628
standard deviation	17199	7124
calculated slope	60793000	4611700
standard deviation	305930	12670

These results (Figure 5.12) establish the assays of NAD^+ and NADH. It should be noted however that gradient 2 failed to separate NAD^+ from one of its -1.1V (and sodium borohydride) reduction products.

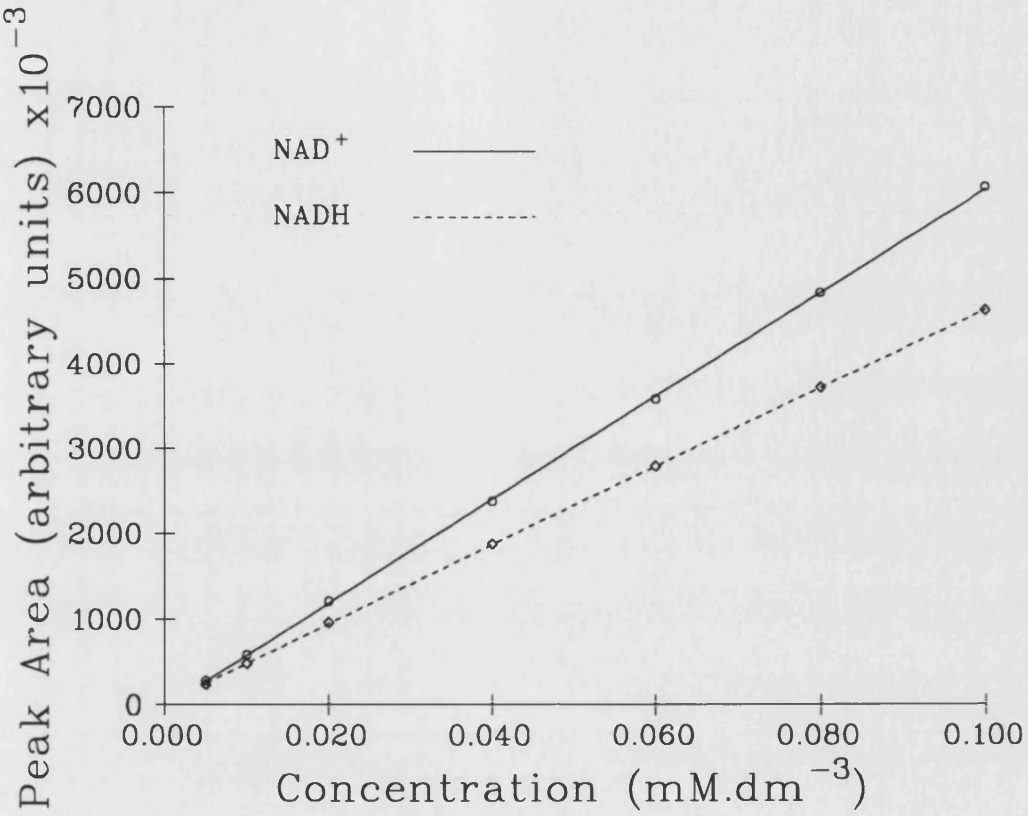


Figure 5.12 HPLC calibration curves for NAD^+/NADH at 260nm

5.2.5.2 NAD^+ starting solution

A typical HPLC trace of the NAD^+ starting solution (nominally 0.1mM) at 260nm is shown below. The one shown is using gradient 2 with the LKB ultrapack column. Typically, this solution was very clean without any significant impurities

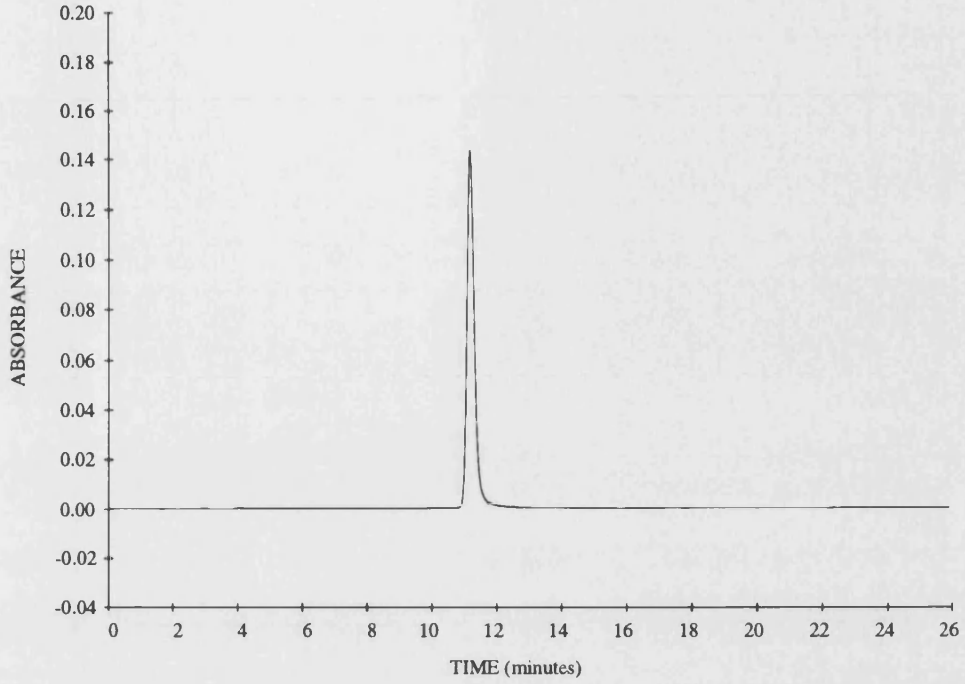


Figure 5.13 NAD^+ starting solution (260nm)

5.2.5.3 NAD^+ and NADH High and Low Calibration Solutions

Typical HPLC traces of the NAD^+/NADH High and low calibration solutions at 260nm are shown below. The high standard solution contained 0.1mM.dm^{-3} of each compound and the low standard contained 0.02mM.dm^{-3} Those shown are from using gradient 2 with the LKB ultrapack column (Figure 5.14 and Figure 5.15). These calibration samples also provided good markers for the positions of peaks in the reduction samples (timings of peaks are more prone to small variations with a gradient system). Two additional significant peaks (at around 3 minutes and 4 minutes on this system) as well as numerable tiny peaks can be seen prior to the NAD^+ peak at around 11 minutes. These impurities were present in the solution prepared from the NADH pre-weighed vials.

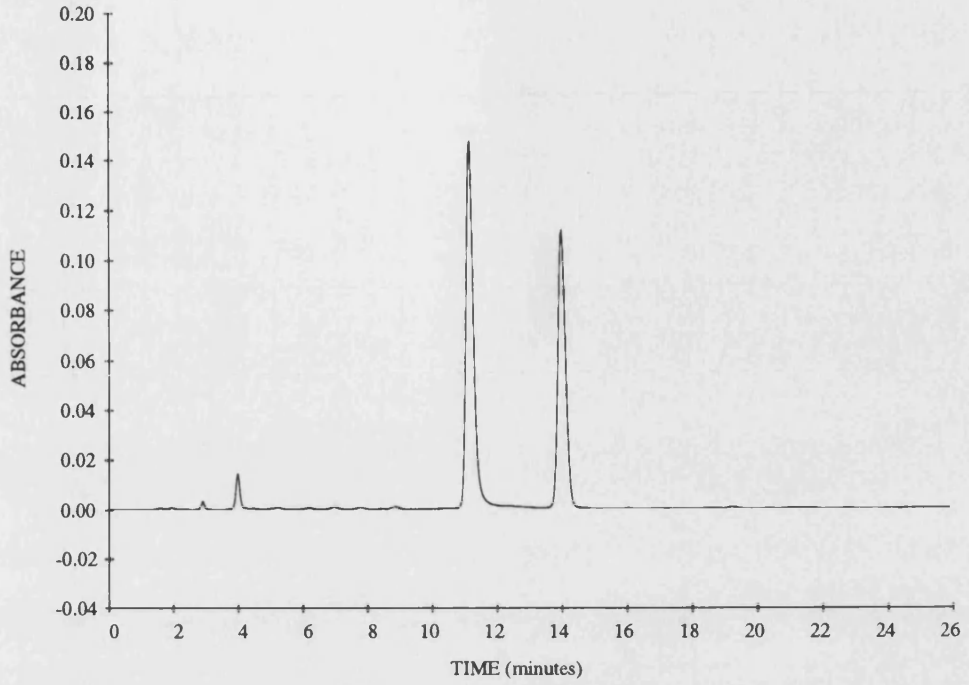


Figure 5.14 NAD^+ and NADH High Calibration Solution (260nm)

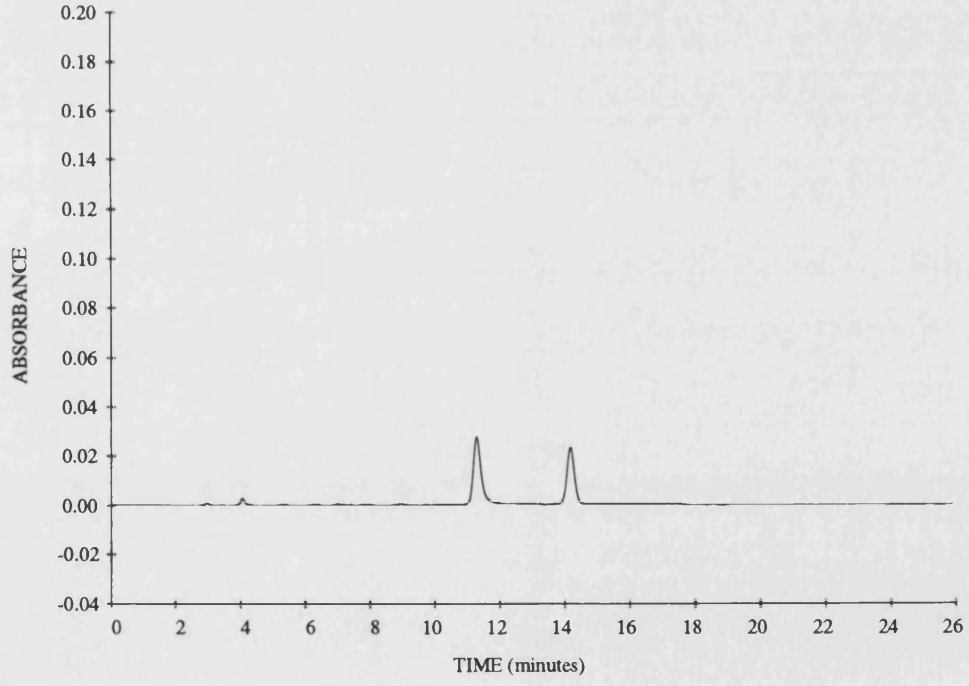


Figure 5.15 NAD^+ and NADH Low Calibration Solution (260nm)

5.2.5.4 Sodium Borohydride reduced NAD^+

The results for the sodium borohydride reduction of NAD^+ appeared to be pH dependent. Figure 5.16 and Figure 5.17 show chromatograms (using gradient 2 with the Chromotech column) from the sodium borohydride reduction of NAD^+ using method 2 outlined previously (pH 9.0). Figure 5.16 shows the typical 3 peaks when the chromatogram is recorded at 340nm (as reported by Jaegfeld[103] and Umeda *et al*[109]) corresponding to the isomers 1,6 NADH (Retention time t_r=4.5 minutes), 1,4 NADH (t_r=5.9 minutes) and 1,2 NADH (t_r=7.1 minutes).

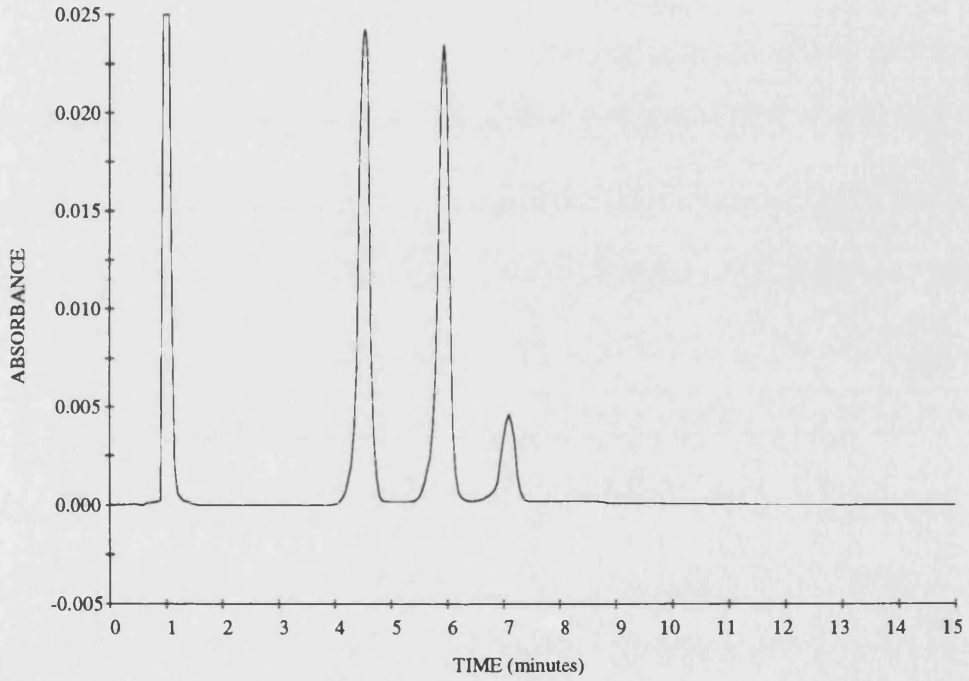


Figure 5.16 Sodium borohydride reduced NAD^+ (reduction method 2, 340nm)

The position of the 1,4 NADH was confirmed from the calibration chromatograms run under the same conditions (not shown). The chromatogram at 260nm (Figure 5.17) shows two additional peaks at t_r=1.7 minutes and at t_r=9.9 minutes. From calibration runs on the same system it was seen that the peak at tr=1.7 minutes was in the same position as the

contaminant seen in the commercial samples of NADH mentioned earlier. The identity of the peak at t_r=9.9 minutes is not known.

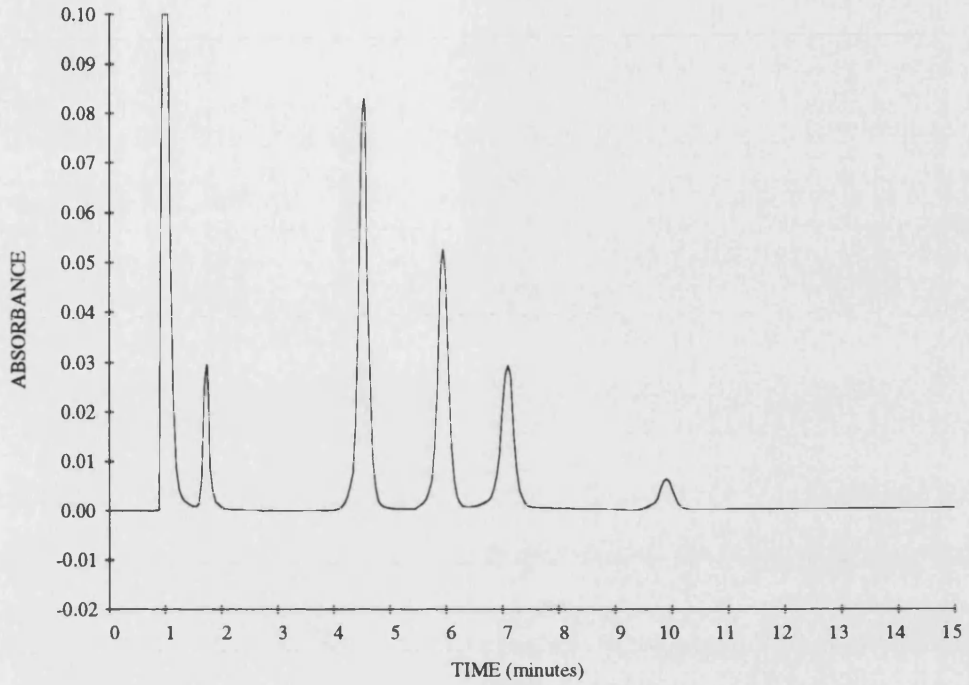


Figure 5.17 Sodium borohydride reduced NAD^+ (method 2, 260nm)

No peaks (apart from the solvent front) were seen when reduction method 2 was performed in exactly the same way but omitting the NAD^+.

When method 3 was used in which the reaction mixture was buffered to pH 7.0 (chromatograms using gradient 2 with the LKB Ultrapak column) the trace at 260nm showed only three major peaks (Figure 5.18) together with the same small early peak (t_r=4 minutes). It is worth at this point referring back to the calibration chromatograms (Figure 5.14 and Figure 5.15) which were run during the same session as this one. These showed $t_r(NAD^+)$=11.3 minutes and t_r(NADP)=14.2 minutes. The first major peak in Figure 5.18 is at 11.5 minutes and was also present on the 340nm trace. This demonstrates that the peak does not represent un-reacted NAD^+ but is probably 1,6 NADH. The second major peak is at 14.2 minutes confirming its identity as 1,4 NADH. The third major peak

occurred at 20.5 minutes and was not present on the 340nm trace. This peak was not identified, but it was not 1,2 NADH which has some absorbance at the higher wavelength. No detectable 1,2 NADH was produced at pH 7.0.

The amount of conversion of NAD^+ to 1,4 NADH produced in these experiments was

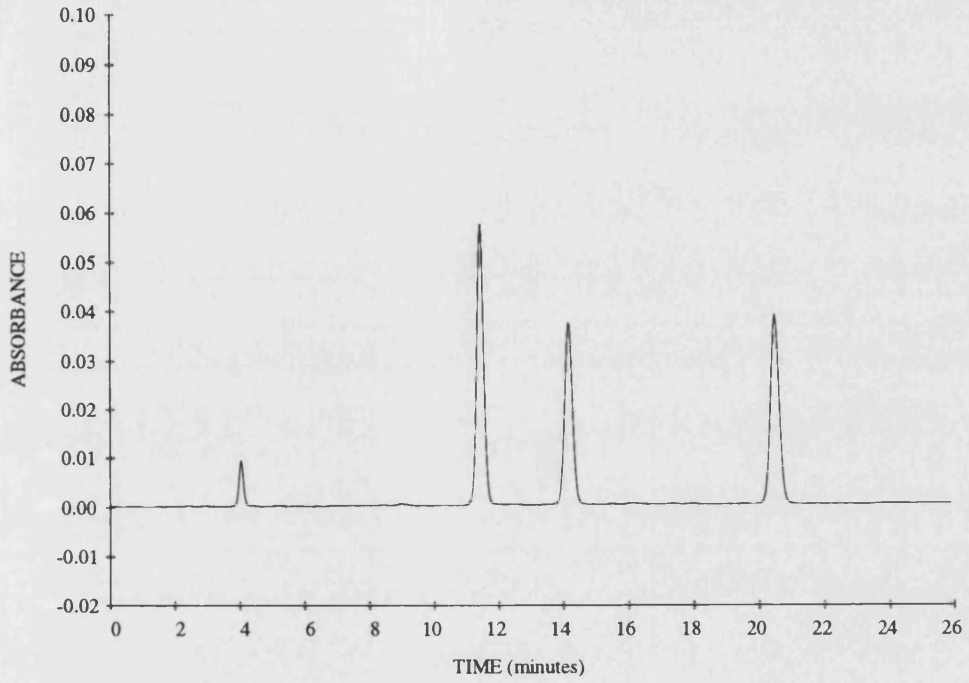


Figure 5.18 Sodium borohydride reduced NAD^+ (method 3, 260nm)

approximately 33% both at pH 9.0 and at pH 7.0 (calculated using integrator peak areas). Please note: The retention times given above are those calculated by the integrator.

5.2.5.5 -1.1V Reduction of NAD^+

The electrolysis buffer background current before the addition of NAD^+ was 0.004mA. On addition of the NAD^+ the current rose to 2.25mA then proceeded to decline slowly. Samples were taken for HPLC analysis at 165 minutes (current = 0.04mA) and at 255 minutes (current = 0.022mA). It was not possible to perform a coulometric analysis for this

experiment because the potentiostat being used required its current meter to be re-zeroed each time the current display range was changed. Re-zeroing could not be performed without disconnecting the cell.

The 260nm chromatograms at each time point are shown below (gradient 2, LKB Ultrapak column).

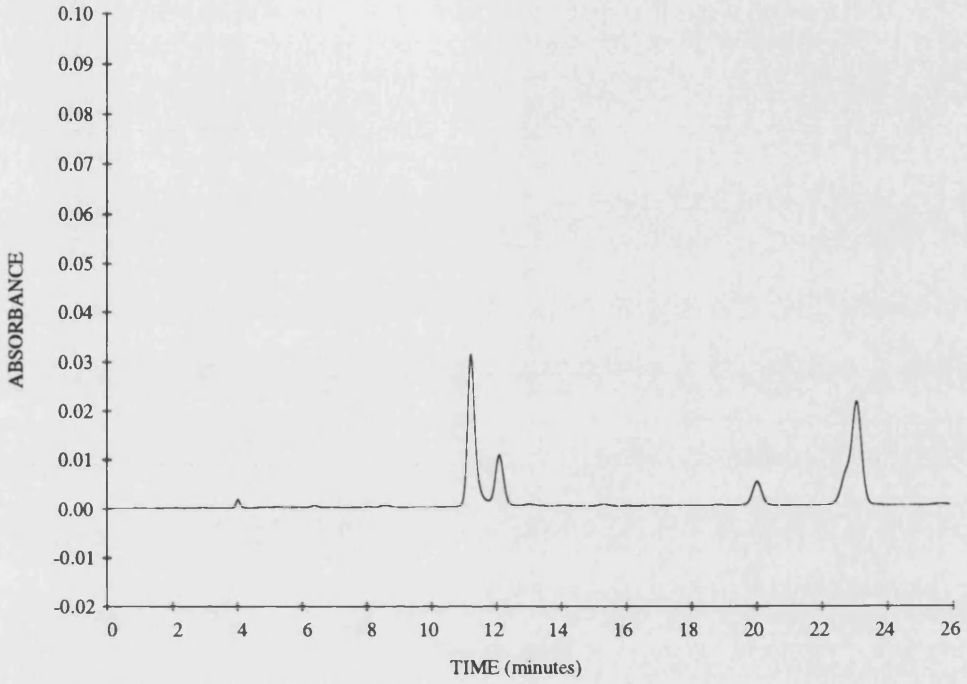


Figure 5.19 NAD^+ Reduced at -1.1V 165 minute sample

Please note the difference in both the absorbance scale and the time scale between these two chromatograms. Figure 5.20 has had its peaks numbered. Additionally, in order to see if any more peaks were going to come off the column the gradient programmer was set to give 100% mobile phase B at t=40 minutes. As the last peak seen was at t_r=31.1 minutes it was demonstrated that this need not have been done.

Peak 1 corresponds to the compound which is seen as a contaminant in commercially available NADH.

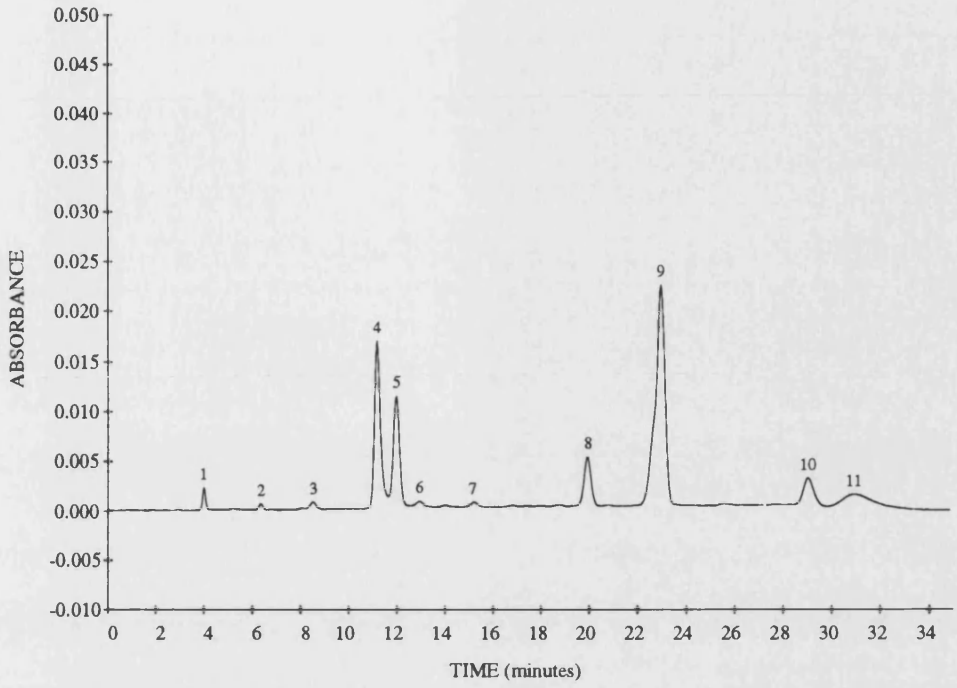


Figure 5.20 NAD^+ Reduced at -1.1v 255 min sample

Summary of peak positions from the Sodium borohydride reduction of NAD^+ and from the NAD^+/1,4-NADH calibration solutions	
Retention time (t_r) in minutes	Peak Identity
4.1	impurity found in commercial NADH and generated during sodium borohydride reduction of NAD^+ at pH 7.0
11.3	NAD^+
11.5	1,6-NADH
14.2	1,4-NADH
20.5	unknown

Peak positions from the -1.1v reduction of NAD^+		
Peak number	Retention time (t_r) in minutes	Peak Identity (where known) and comments
1	4.1	see above table
2	6.4	
3	8.5	
4	11.3	residual NAD^+
5	12.1	
6	13.0	
7	15.1	
8	20.0	
9	23.0	
10	29.1	
11	31.0	

Although many small ripples in the baseline can be seen between these main peaks, one of the most important features of this data is the complete lack of any peaks corresponding to 1,6-NADH or 1,4-NADH (and probably 1,2-NADH). The large number of other peaks supports the work of Jaegfeldt[103] who suggested the reaction produced numerous dimers and who attempted semi-preparative HPLC in order to try and identify these peaks.

5.2.5.6 -1.8V reduction of NAD^+

The electrolysis buffer background current before the addition of NAD^+ was considerably higher than the -1.1V reduction, as might be expected, at 0.84mA. On addition of the NAD^+ the current rose to 23mA then proceeded to decline at a somewhat faster rate than previously. The reaction was stopped when the current had reduced back to the

background level (140 minutes). The chromatogram at 260nm shown below (Figure 5.21) was obtained using gradient 1 with the Chromotech column.

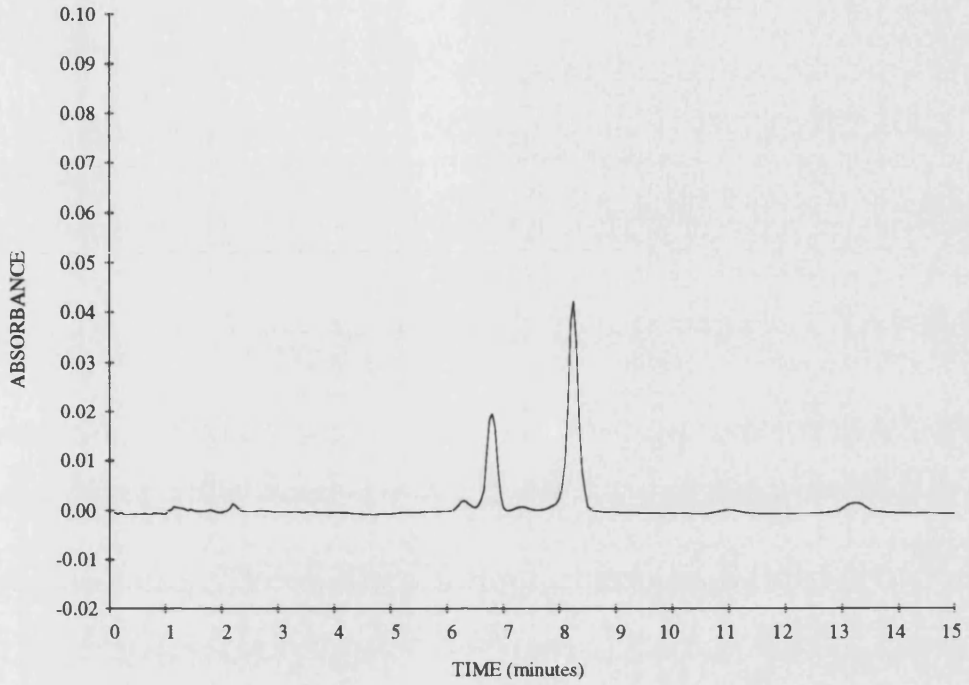


Figure 5.21 -1.8V Reduction of NAD^+ (Gradient 1)

From the chromatograms of the sodium borohydride reduction of NAD^+ and the NAD^+/NADH calibration solutions using gradient 1, the peak at t_r=6.8 minutes corresponds with 1,6-NADH and the peak at t_r=8.2 minutes corresponds with 1,4-NADH. A small amount of un-reduced NAD^+ can be detected at t_r=7.3. A range of other peaks can be seen on the chromatogram which were not identified. Ideally, the -1.8V reduction should have been re-performed and analysed using gradient 2 and the LKB Ultrapak column to allow direct comparison with the -1.1V reduction.

5.2.6 Conclusions

The reduction of NAD^+ at a mercury electrode produces a complex mixture of reaction products. The chromatographic method described, particularly using gradient 2 with the

LKB Ultrapak column, provides a useful system for the investigation of the influence that any mediators may have on the products if they are included in the reaction mixture.

6 RHODIUM

6.1 Introduction

Various organometallic complexes of rhodium have been used to try and mediate the electrochemical reduction of $NAD(P)^+$ to NAD(P)H. The work in this area has been driven by the need to be able to regenerate this cofactor in biochemical reactors, and their use as potential mediators in biosensors has not really been considered in the literature.

The first evidence that certain polypyridine rhodium complexes might be able to mediate cofactor reduction came from Wienkamp and Steckhan[110] who formed complexes of rhodium with 2,2′–bipyridine (bpy). The structure of bpy is given below (Figure 6.1).

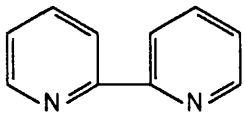


Figure 6.1 The structure of 2,2′-bipyridine

They showed that rhodium(III)-tris(2,2′–bipyridine)$^{3+}$ ($[Rh(bpy)_3]^{3+}$) was able to generate rhodium(I)–bis(2,2′–bipyridine)$^{1+}$ ($[Rh(bpy)_2]^{1+}$) at a cathode poised at approximately -0.9V *vs* Ag/AgCl electrode. This secondary complex is able to transfer two electrons (together with a hydrogen ion supplied from the solution) to NAD^+, generating NADH. Their proposed reaction scheme is shown in Figure 6.2.

They were able to regenerate NAD^+ using horse liver alcohol dehydrogenase (HLADH) to catalyse the reduction of cyclohexanone showing that, in the main, the NADH produced was active (i.e. 1,4–NADH). They were able to measure the total quantity of cofactor dimer formed and calculated that 0.9% of the NAD^+ ended up as dimer per cycle.

Rhodium has an atomic number of 45 and in its ground state it has 7 electrons in its d orbital. In its 3+ state it has a d^6 configuration and will accept electron pairs from up to 6 nitrogen atoms. Thus, rhodium is able to form an organometallic complex with three bpy

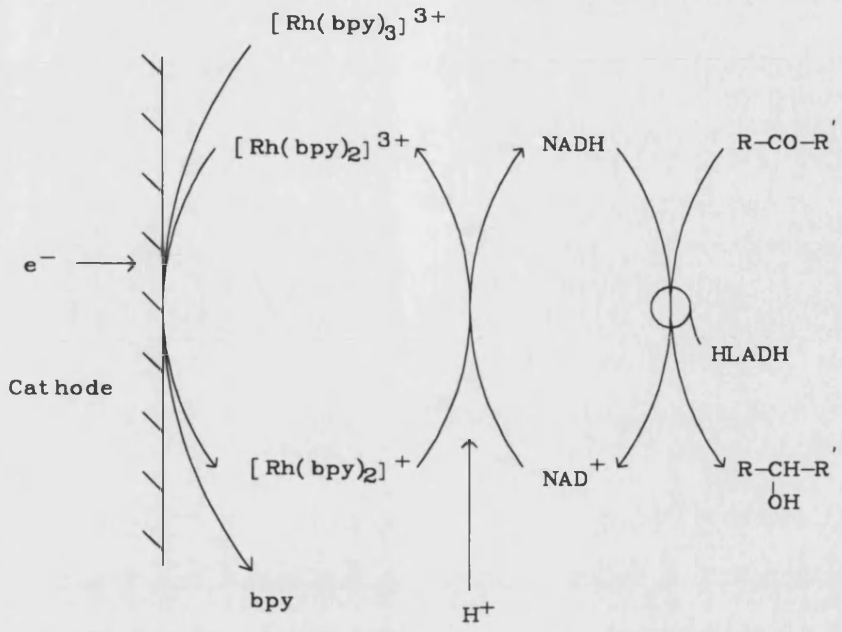


Figure 6.2 Regeneration of NADH from NAD^+ by electrochemically generated $[Rh(bpy)_2]^+$ as an electron transfer agent (from ref 110)

molecules.

When the rhodium atom is reduced to its 1+ oxidation state, one nitrogen must dissociate from the complex.

In the reaction scheme given above, the reduction leads to the dissociation of a complete bpy ligand and therefore the dissociation of two nitrogens. This set of events leads to some of the problems associated with rhodium(III)-tris(2,2′–bipyridine)$^{3+}$ as an NAD^+ reduction mediator. Other potential ligands in the solution will coordinate with the rhodium. It was reported in the paper by Wienkamp and Steckhan[110] that eventually the current at the electrode reduces due to fouling of the graphite cathode used by $[Rh(bpy)_2(H_2O)_2]Cl$ or $[Rh(bpy)_2(OH)_2]Cl$.

Umeda *et al* [111] attempted to improve the stability of this type of compound by using two terpyridine ligands (Figure 6.3) instead of three bipyridine ligands.

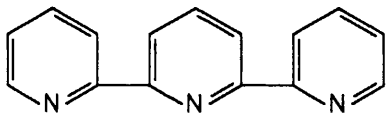


Figure 6.3 The structure of 2,2′;6′,2″-terpyridine (terp)

This is a logical move because if the rhodium is reduced to Rh(I) and a nitrogen has to dissociate from the complex, because the ligand is attached by the coordination of two other nitrogens, the ligand as a whole may remain in place. If subsequently the rhodium is re-oxidised to Rh(III) the dissociated nitrogen will be in the correct position to re-associate with the atom in preference to other potential ligands in the solution.

They examined this compound for its ability to catalyse the photochemical reduction of NAD^+ and found it to be a good catalyst for the production of 1,4-NADH. Its electrochemical behaviour was not examined.

Ruppert *et al*[112] described a system where formate was used to supply the 2 electrons and a proton required to reduce $NAD(P)^+$. This reaction was coupled using pentamethyl-cyclopentadienyl rhodium complexes formed with three different bpy based ligands (bpy itself or a dimethyl-bpy compound, either 4,4′-Me_2bpy or 6,6′-Me_2bpy). In this system it was postulated that reduction of the rhodium from III to I did not occur, but that the compound formed a hydride intermediate (Figure 6.4).

They suggested that the rhodium compound initially formed a complex with the formate anion which was then followed by the extrusion of CO_2. This interaction with formate would appear to be crucial for this compounds activity and use as an electrochemical mediator would seem unlikely (or would have to occur by completely different mechanisms).

From the reduction experiments of NAD^+ at mercury electrodes it has been demonstrated that at -1.1V *vs* S.C.E. the majority of the cofactor is converted into a range of dimers. At the more negative electrode potential of -1.8V, hydrogenation does occur but different

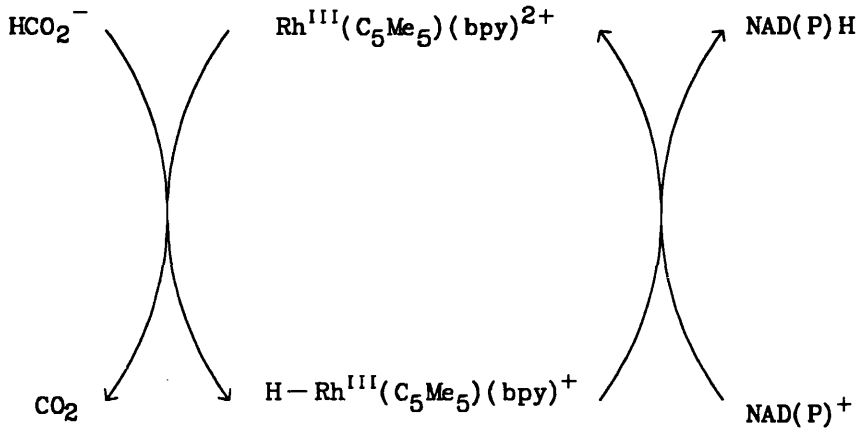


Figure 6.4 Reduction of $NAD(P)^+$ with formate catalysed by the rhodium complexes described in the text (from reference 112)

positional isomers are produced (a mixture of 1,2 1,4 and 1,6 NADH) as well as the dimers seen at the less cathodic potential. At -1.8V, a mercury electrode will start to electrolyse the water in which the experiment is taking place and hydrogen gas is evolved from the electrode surface. It would therefore seem likely that both a hydrogen radical and a NAD radical are being produced at the electrode surface and the range of products produced is based on the probabilities of collision:-

Hydrogen radical + Hydrogen radical	→	Hydrogen (gas)
Hydrogen radical + NAD radical	→	NADH (1,2 1,4 or 1,6)
NAD radical + NAD radical	→	Dimers

One of aspects of the mechanism by which rhodium compounds may help as mediators may be by lowering the potential required for the production of hydrogen radicals.

Cosnier *et al* [113] have used a pyrrole derivative of bpy as a ligand to form a complex with rhodium and pentamethylcyclopentadiene. The pyrrole was then electro-polymerised at an anodic potential on to either glassy carbon, carbon-felt or platinum. They were able

to generate hydrogen gas at -0.55V *vs* Ag/AgCl (pH 1.0) using coated carbon felt electrodes (a process which does not occur at un-modified electrodes).

It is important from the point of view of biosensor development that yields of active 1,4-NADH are as high as possible. With this aim in mind it is important that these compounds are examined using an experimental design which can detect all the products of the reduction. Some of the papers in the literature assess the success of the compound by examining the absorbance increase at 340nm. This does not give the complete picture because the inactive compounds 1,2-NADH and 1,6-NADH have some absorbance at 340nm. It would be beneficial, when the percentage of active 1,4-NADH decreases with each cycle, to examine the inactive products of the reaction using techniques such as the HPLC system described in chapter 5.

With this aim in mind it was decided to synthesize some potential mediators and examine their behaviour at a mercury electrode.

6.2 Synthesis of Rhodium complexes

The two basic methods are available in order to form two different series of rhodium complexes. These are outlined below.

6.2.1 Reactions of pentamethylcyclopentadienylrhodium chloride dimer

Pentamethylcyclopentadienylrhodium chloride dimer (Figure 6.5) will react with various nitrogen containing ligands, to form complexes of the form:-

$$[CpentRh^{III}(L_1)(L_2)]^{n+}$$

where Cpent is cyclopentadiene, L_1 is a ligand containing two nitrogens which have lone pairs capable of coordination with the rhodium, L_2 is H_2O, Cl^-, OH^-. The overall charge is dependent upon L_2 and can be 1, 2 or 3+.

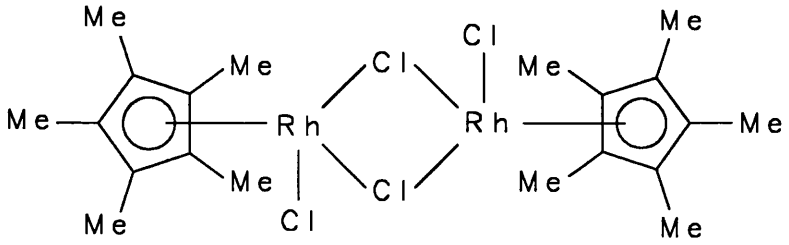


Figure 6.5 The structure of pentamethylcyclopentadienylrhodium chloride dimer

An attempt was made to form a range of compounds based upon the reaction methodology outlined by Kölle and Grützel[114], although the experimental details in this paper were not comprehensive. The method used was also influenced by the work of Cosnier *el al*[113], who reacted the dimer with a slight excess of [bis(pyrrol-1-ylmethyl)methoxycarbonyl-2-2′-bipyridine in ethanol.

6.2.1.1 Chemicals

2,2′-dipyridyl (Bipyridine), 1,10-phenanthroline, biquinoline, 4,4′-dimethyl-2,2′-dipyridyl and Pentamethylcyclopentadienylrhodium chloride dimer were obtained fron the Aldrich Chemical Company Ltd.

All solvents were of HPLC grade as listed earlier. RO water was used throughout

6.2.1.2 Method

For this work, 0.3mmol (185mg) of the dimer was reacted with 0.72mmol of the appropriate ligand in 10ml of methanol. Using this solvent, the dimer is practically insoluble (although it colours the solution deep red) but it dissolves as it reacts to form the more soluble product.

The ligands used were:-

2,2′-dipyridyl (Bipyridine)	112mg	(see Figure 6.1 for structure)
1,10-phenanthroline	130mg	

biquinoline	184mg
4,4′-dimethyl-2,2′-dipyridyl	133mg

Completion of the reaction was indicated by all the dimer dissolving and by a colour change (all solutions became orange). The solution was then evaporated to dryness in a rotary evaporator (at room temperature) and redissolved in a minimum amount of warm (35°C) 50% aqueous ethanol, followed by cooling to around 20°C.

In hindsight, it was a mistake to use such a large excess of ligand because whilst trying to crystallise the products (all water soluble) the excess ligand (low water solubility) came out of solution first. 0.3mmol of dimer should probably have been reacted with 0.6mmol of ligand.

Attempts were made to recover the situation by evaporating the solutions to dryness again followed by dissolving them in 2ml of methanol. Ether was then added until the product just began to precipitate (ligands ether soluble). Gentle warming was then used to re-dissolve, followed by leaving the solution for crystals to form.

6.2.1.3 Results

Elemental analysis of the crystals collected were performed in the School Of Chemistry.

Ligand	Element	Expected percentage	Found percentage
2,2′-Dipyridyl	N	5.80	5.66
	C	49.7	51.8
	H	5.21	5.06
1,10-phenanthroline	N	5.52	5.39
	C	52.09	51.8
	H	4.96	5.06
Biquinoline	N	4.80	4.54
	C	57.65	58.7
	H	5.01	5.48
4/4′-dimethyl-2,2′-dipyridyl	N	5.47	5.22
	C	51.68	50.2
	H	5.71	5.67

As can be seen from this data, the elemental analysis of these compounds gave values which were a considerable way out from expected values. It would not even be possible to tell the compounds apart from this data.

The reasons for this presumably lie in the crystallisation technique which was probably inappropriate. The expected values in the above table are calculated on the basis of L_2 being H_2O. Although the compounds had all been exposed to water during the first attempt at crystallisation, the solvent system used for the second attempt was non-aqueous. In this solvent system, it may be possible that the lone pair of electrons on the oxygen of diethyl ether may be able to interact with the rhodium.

It is believed that if these compounds were re-made with a lower excess of ligand, followed by crystallisation from an aqueous or part aqueous solvent system, the elemental analyses would have been better. Other methods of structure elucidation, such as ^{1}H-NMR or f.a.b. mass spectroscopy, were not considered appropriate until this had been done.

6.2.2 Reaction of rhodium chloride with the ligands bpy and terp

It was intended to attempt the synthesis of the following three compounds, according to published methods. This work was unfortunately not completed.

1) $Rh^{III}(bpy)_3Cl_3$

2) $Rh^{III}(terp)_2Cl_3$

3) $Rh^{III}(terp)(bpy)Cl_3$

The papers containing the intended methods are given for reference.

6.2.2.1 Method - Synthesis of $Rh^{III}(bpy)_3Cl_3$ and $Rh^{III}(terp)_2Cl_3$

In the past, rhodium chloride has been fused with these ligands (Harris and McKenzie[115] and Martin and Waind[116]) in order to form appropriate complexes. More recently, a simpler method has been developed by Kirch *et al* [117] which involves heating rhodium chloride with the appropriate ligand in 50% aqueous ethanol in the presence of N-ethyl morpholine. The disappearance of the ligand during these reactions can be monitored (to determine an end point) by examining the UV spectra of a diluted sample of reaction mixture.

6.2.2.2 Method - Synthesis of $Rh^{III}(terp)(bpy)Cl_3$

The synthesis of this compound relies first upon the synthesis of $Rh^{III}(terp)Cl_3$ according to the method developed by Bhayat and McWhinnie [118]. This involves the refluxing of

rhodium chloride and the ligand (at equimolar ratios) in a 20% water 80% ethanol solvent system.

Once this compound has been collected by crystallisation it can be reacted with the bipyridine ligand (reflux for 6 hours in 25% dimethylformamide 75% ethanol) as outlined by Frink *et al* [119]

7 FINAL DISCUSSION

7.1 Creating A Biosensor

This thesis has illustrated that considerable progress will be necessary in the future to enable biosensors based on microbial ketone reductases to work effectively. However, many of these advances may not be too far away. Some of the current problems which need to be overcome are discussed here by describing the components which will be required to create such a biosensor.

Firstly, a suitable electrode surface is required. Although it is difficult to chemically attach enzymes and mediators to thin film metal electrodes, the manufacturing advantages to be gained by integration of the sensor with the necessary electronics are worth pursuing.

As with biosensors based on oxidative reactions, it should be possible to use compounds which will undergo electropolymerisation to entrap the enzyme. The polymer chosen should be able to conduct at cathodic potentials.

Electrode platinisation[35] provides another technique by which enzyme entrapment can be achieved and by which the effective electrode surface area can be dramatically increased, leading to enhanced current densities. This method should be compatible with thin film electrodes, but the low pH of the chloroplatinic acid used in platinisation may reduce or destroy enzyme activity.

Metal electrodes may have a considerable disadvantage though. The potential at which the electrolysis of water can occur is less negative at these electrodes than is necessary at some of the other electrode materials[14].

Secondly, a keto reductase with suitable specificity for the analyte needs to be found and isolated. As has been seen, microorganisms can be screened for an ability to carry out the reduction of ketone containing compounds. If one is found, it ought to be possible to grow the microorganism in large enough quantities using a fermenter to allow enzyme

extraction. The extension to this is the possible determination of the structure of the enzyme and perhaps expressing the gene for the protein in a bacteria such as *E. coli* to allow large amounts to be produced. Although biosensors can be created using immobilised intact bacteria, these have poorer response times and biocompatibility problems. Sensors based on pure immobilised enzyme will prove to be much more effective devices.

The third requirement for such a sensor is the development of a reaction modifier/mediator which is able to be oxidised by the enzyme's reduced cofactor (regenerating active cofactor), and is then able to be reduced at the electrode surface (regenerating itself).

7.2 Future Work

This thesis has outlined many of the developments in biosensor technology which will be required in order to create a warfarin sensor. In addition, some of the research methodologies which will be necessary in the quest for some of these advances have been developed. This section describes some of the work which was planned but not done due to lack of time, and some of the potential experiments which would have followed on from this.

7.2.1 Warfarin

An effective HPLC assay for warfarin and its metabolites in bacterial samples is essential to the continued work towards the extraction of any warfarin reductase. The HPLC of warfarin using the pH 2.8 mobile phase outlined in section 4.4.3.1 gave particularly good separation of the warfarin alcohols. However, this assay is likely to become more difficult to perform (due to interfering compounds) once the bacterial sample has been treated in order to attempt enzyme extraction.

In order to improve the assay, the normal phase preparative HPLC method given in this thesis should be used to provide individual samples of each of the four warfarin alcohols

for use as standards. In addition to this, an internal standard should be found which elutes in between the warfarin alcohol peaks and the warfarin peak.

7.2.2 The Warfarin Reductase

It has been shown in this thesis that ATCC 19140 and ATCC 19070 are able to reduce the ketone group found in the warfarin molecule. The purification and extraction of the enzyme responsible would be the next goal.

From the high cell mass obtained using a fermenter, various cell disruption techniques would be tried in an attempt to liberate the enzyme[120,121] whilst attempting to retain as much of the enzyme activity as possible. These might include the use of a French press or other homogeniser to break down the bacterial cell wall. Another approach might be to use enzymatic breakdown of the cell wall using lysozyme. The reductase activity remaining after each process attempted would need to be measured (probably with a standard blend of different cofactors added to the mixture) together with some estimate of the degree of cell disruption. Centrifugation of the homogenate followed by the measurement of enzyme activity in each of the fractions would then need to be performed. The methodology required from this point on would depend upon the location any enzyme activity.

If the enzyme were to be successfully isolated a whole set of experiments would need to be performed including the determination of the optimum pH and temperature for enzyme activity. The effect of the presence or absence of a range of cofactors would need to be studied in order to determine which cofactor is required by the enzyme. The specificity of the enzyme for warfarin would have to be tested.

7.2.3 NAD(P)H

This thesis has outlined a suitable method for the electrochemical reduction of NAD(P) and for the HPLC determination of the product profile from such an experiment.

If any potential reaction modifiers/mediators were to be tested, the HPLC methodology may need to be adjusted to take in to account the chromtographical behaviour of that

mediator. As mentioned previously, the measurement of the amount of active NAD(P)H generated cannot be achieved by using simple absorbance measurements.

7.2.4 Rhodium based mediators

As has been seen, various rhodium complexes have been shown in the literature to be able to modify the products of electrochemical nicotinamide cofactor reduction. These compounds are not perfect however, and their mechanisms of action are unclear.

The synthesis and electrochemical testing of a wide range of rhodium compounds with nitrogen heterocycle ligands needs to be carried out. The methodology for such syntheses are becoming more widely published. Anyone attempting this type of reaction for the first time will come to appreciate that the high cost of rhodium compounds will mean that they will be trying to attempt recrystallisations from very small fluid volumes. Any methodology needs to take this into account. One suggestion is to form crystals in a small vessel which has a 0.2μm membrane filter incorporated in to its base. Such a filter will not allow liquid through it without the use of pressure, and once crystals have formed, the filtrate can be removed by applying a vacuum to the bottom of the filter.

Some encouraging articles on the use of rhodium compounds have appeared in the recent literature.

Substituted 2,2′-Bipyridyl complexes of pentamethylcyclopentadienyl rhodium chloride have been further studied by Steckhan *et al*[122]. This group have started to examine the influence that substituent groups (on the bipyridyl moiety) have on their ability to reduce nicotinamide cofactors.

Work on Rhodium compounds containing a pyrrole derivative of bipyridyl (which is subsequently polymerised at the electrode surface) has continued[123] leading to the production of an electrode for the reduction of pyruvate. They were unable to detect any dimer formation during bulk electrolysis of pyruvate using this electrode.

Umeda *et al*[124] have started to elucidate how NAD^+ and $Rh(terp)_2^{+3}$ interact at a molecular level. The results of this work may lead to an improved series of compounds.

Ryabov *et al*[125] in a preliminary report, have succeeded in complexing NAD^+ directly with pentamethylcyclopentadienyl rhodium chloride.

One of the problems to be overcome with the use of reaction modifiers/mediators in biosensors is whether one can be found which works at a sufficiently small negative potential (this problem does not affect these compounds ability to work in biochemical reactors to the same extent). If these compounds require a large negative potential, many other compounds will be able to be reduced at the electrode surface, producing a large background current. As mentioned earlier, this may include electrolysis of water and the reduction of dissolved oxygen. Although it may be possible to correct for some of the interfering compounds by subtracting the current from a second electrode which does not have the enzyme present, mediators with less negative E_0 values are desirable.

E_0 and k_s values would need to be determined for all potential mediators in order that structure modifications can be fully assessed (analogous to the development work for the ferrocenes used in glucose sensors).

The synthesis of novel ligands which have side groups attached (perhaps with suitable spacing molecules - see FAPP and FAPAPP in figure 1.9) to allow chemical attachment to the electrode, to the enzyme or to the cofactor could be attempted.

Beley and Collin[126] have already generated a pyrrole derivative of terpyridine and used this to form complexes with rhodium chloride. Electrodes modified with these complexes were able to produce 96% active NADH from NAD^+ at -750mV.

Any successful compounds would need to be tested for toxicity if their use in any implantable sensors were to be considered.

7.2.5 Other Mediators

Bergel and Comtat[127] have managed to use the flavin cofactors $FADH_2$ and $FMNH_2$ to reduce $NAD(P)^+$. This reaction is thermodynamically unfavourable but was achieved at a potential of -650mV by using a large electrode surface area together with a very small solution volume (conditions which can be achieved using capillary fill devices). An assessment of any other reduction products (e.g. using HPLC) was not made.

Davies *et al*[128] have looked at the possibility of generating synthetic compounds which will mimic the cofactor activities of NAD/NADH whilst removing the problems associated with the reduction of NAD. If such compounds can be realised, they might work as both cofactor and mediator.

7.3 The Future for Amperometric Biosensors

The amperometric glucose sensor was the first biosensor to make the headlines. The revolution in analyte measurement that appeared to be heralded by these sensors has not really happened. The reason for this is probably that in many ways the glucose sensor has proved to be the simplest sensor (technically) to create for a clinically useful analyte. Few molecules which you would wish to measure the levels of have enzymes which act upon them with such high specificity. Even fewer have enzymes which are readily available from biochemical suppliers.

The world of biosensor research also seems to have occupied its thoughts with cheapness of sensors and with mass production. This is perhaps because of comparisons with the needs of the glucose sensor marketplace. There are a variety of medical situations where biosensors would be useful and would be cost effective, even if the actual sensor cost were to be in pounds rather than in pence. These might include such measurements as drug levels by ambulance crews when presented with an overdose case, or the sensing of biochemical changes produced by the body in acute illness such as stroke or myocardial infarction, allowing the administration of disease modifying drugs appropriately and

quickly. It might be possible to measure the level of drug molecules in intensive care situations with direct feedback to drug administration pumps. They could allow rapid monitoring of levels of drugs with narrow therapeutic indices, reducing possible toxicity.

One question which needs to be asked of the medical professions is; if you could measure the level of any compound what would you actually want to measure. There are a range of compounds which they would like to measure but are currently unable to measure at all or are unable to measure on a sufficiently short time scale for the measurement to be of any use.

Very few real breakthroughs in biosensor design appear to have been published recently. The reasons for this lack of progress have been addressed in a review article by Griffiths and Hall[129]. Organometallic rhodium compounds may provide a path towards the reliable electrochemical regeneration of reduced nicotinamide cofactors. This in turn may open up the world of amperometric biosensors to a much extended range of enzymes.

Most of the researchers in the field of rhodium chemistry are looking at these compounds with a view towards use in biochemical reactors. Only one of the papers on rhodium cited in this thesis makes any mention of a possible role in biosensor devices.

The search for compounds which will mediate the effective electrochemical regeneration of nicotinamide cofactors is worthy of considerable research effort.

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APPENDIX ONE

Voltammetry Data Collection Program

```
 10 REM >:0.$.CYC2806A
 20 REM      ====================================================
 30 REM      DATA COLLECTION PROGRAM - GRJ Harwood - 6/90   v.1.2
 40 REM      ====================================================
 50 :

 60 REM ------------------------------------------------------------------------
 70 REM                          INITIALISATION
 80 CLS:
    CLG:
    MODE8:
    VDU 28,0,31,79,27
 90 *MOUNT 0
100 COLOUR 131:
    COLOUR 1:
    GCOL 131:
    GCOL 0
110 CLEAR:
    SET=-1:
    FS$="":
    textwidth=16:
    textheight=16:
    IV=0:
    FV=0:
    MV%=0
120 DIM N(4,50),T(2,50),T$(50),H$(27),S(2,15),D(1,30000)
130 DIM SF%(10,1),m%(100),M1%(4,23),T1$(11),T1(4,11),T2(4),CL$(4)
140 DIM F(1,1000),R(1,1000)
150 PROCcode
160 ON ERROR PROCerror
170 PROCheader
180 :

190 REM ------------------------------------------------------------------------
200 REM                           MAIN MENU
210 REPEAT
220 CLS:
    INPUT"(F)ixed voltage, (L)inear sweep, (C)yclic sweep or (E)dit" ;FS$
230 CASE FS$ OF
240 WHEN "F","f"
250 FS$="F"
260 TYPE$="FIXED VOLTAGE"
270 PROCfixed
280 WHEN "L","l"
290 FS$="L"
300 TYPE$="LINEAR SWEEP VOLTAMMETRY"
310 PROCsweep
320 WHEN "C","c"
330 FS$="C"
340 TYPE$="CYCLIC SWEEP VOLTAMMETRY"
350 PROCsweep
360 WHEN "E","e"
370 FS$="E"
380 PROCedit
390 VDU 28,0,31,79,27
400 ENDCASE
410 UNTIL ERR=17
420 END
430 :

440 REM ------------------------------------------------------------------------
450 DEF PROCfixed
460 B%=0:
    m%=0
470 PROCholdinput
480 PROCscale
490 PROCzero
500 PROCholdaxis
510 CLS:
    PRINT"PRESS ANY KEY TO START":
    IFGET
520 interval=(TS*60/199)*100:
    REM TAKE 200 POINTS DURING TS MINUTES
530 TIME=0
540 FINISHTIME=TIME+interval
550 Z$="":
    PROCread
560 IF Z$="E" THEN 610
570 REPEAT
580 CLS:
    YN$="":
    INPUT"Do you want to reset variables  Y or N";YN$
590 UNTIL YN$="Y" OR YN$="N"
600 IF YN$="Y" THEN 470 ELSE 500
610 CLG:
    B%=B%-1:
    PROCholdaxis:
    PROCscan
620 REPEAT
630 CLS:
    CS$="":
    INPUT"Save (D)ata, (S)can or (E)xit";CS$
640 UNTIL CS$="D" OR CS$="S" OR CS$="E"
650 IF CS$="D" THEN
660 GOTO 770
670 ELSE IF CS$="S" THEN 730
680 ENDIF
690 REPEAT
```

```
700 YN$="":
    INPUT"Are you sure Y or N";YN$
710 UNTIL YN$="Y" OR YN$="N"
720 IF YN$="N" THEN 620 ELSE 790
730 FOR A%=1 TO 10
740 SF%(A%,0)=0:
    SF%(A%,1)=0
750 NEXTA%
760 PROCholdselect
770 PROCsave
780 GOTO 610
790 ENDPROC
800 :

810 REM ------------------------------------------------------------------------
820 DEF PROCsweep
830 PROCpointer_reset
840 PROCholdinput
850 PROCsweepinput
860 PROCscale
870 PROCzero
880 PROCholdaxis
890 *FX 200,1
900 CLS:
    PRINT"PRESS ANY KEY TO START":
    IFGET
910 *FX 200,0
920 interval=(TS*60/199)*100:
    REM TAKE 200 POINTS DURING TS MINUTES
930 TIME=0
940 FINISHTIME=TIME+interval
950 Z$="":
    PROCread
960 IF Z$="S" THEN 990
970 PROCholdinput
980 PROCzero:
    GOTO 880
990 CLG:
    PROCsweepaxis:
    IF m%<1 THEN 1030
1000 FOR a%= 1 TO m%
1010 PROCredraw2
1020 NEXT
1030 CLS:
     PRINT"Press any key to START":
     IFGET
1040 SYS6,146,4,Z% TO ,,Z%
1050 Z$="":
     jump%=0
1060 PROCread2
1070 IF Z$="E" THEN 1340
1080 REPEAT
1090 CLS:
     RS$="":
     L%=0
1100 INPUT"Reset (A)ll variables, Sweep (R)ate, (S)cale or re-use (O)ld values";RS$
1110 CASE RS$ OF
1120 WHEN "A","a"
1130 PROCsure
1140 IF YN$="Y" THEN L%=2 ELSE L%=1
1150 WHEN "R","r"
1160 REPEAT
1170 CLS:
     INPUT "NEW SWEEP RATE (mV per second)";MV%:
     PRINT
1180 UNTIL MV%<>0
1190 CL$(2)=CL$(2)+", "+STR$(MV%):
     L%=3
1200 PROCerase
1210 WHEN "S","s"
1220 PROCsure
1230 IF YN$="Y" THEN
1240 PROCpointer_reset
1250 L%=4
1260 ELSE
1270 L%=1
1280 ENDIF
1290 WHEN "O","o"
1300 L%=3:
     PROCerase
1310 ENDCASE
1320 UNTIL L%<>0
1330 ON L% GOTO 1340,830,880,860
1340 REPEAT:
     CLS:
     CS$=""
1350 INPUT"Save (D)ata, (R)eturn or (E)xit";CS$
1360 CASE CS$ OF
1370 WHEN "D"
1380 PROCsave
1390 WHEN "E"
1400 PROCsure
1410 IF YN$<>"Y" THEN CS$=""
1420 ENDCASE
1430 UNTIL CS$="R" OR CS$="E"
1440 IF CS$="R"THEN 1080
1450 ENDPROC
1460 :

1470 REM ------------------------------------------------------------------------
1480 DEF PROCholdinput
1490 REPEAT
1500 CLS
1510 INPUT"HOLD VOLTAGE";HV:
     PRINT
1520 UNTIL HV>=-1 AND HV<=1
1530 REPEAT
1540 INPUT "TIMESCALE OF HOLD PERIOD in minutes";TS:
     PRINT
1550 UNTIL TS>=2.8 AND TS<=10
1560 ENDPROC
1570 :
```

```
1580 REM ---------------------------------------------------------------------
1590 DEF PROCzero
1600 @%=&20205
1610 CLS
1620 PRINT"  Set the 626 off-hold-sweep switch to OFF - adjust Icomp until the reading"
1630 PRINT"       is as close to zero as possible (with the potentiometer locked)"
1640 PRINT "                            Press any key when set"
1650 VAR2%=5000
1660 REPEAT
1670 store%!12=VAR2%
1680 CALL code%
1690 Y%=store%!4
1700 PRINT TAB(30,4);(Y%/VAR2%)-2048;"              ";
1710 UNTIL INKEY$(10)<>""
1720 ENDPROC
1730 :

1740 REM ---------------------------------------------------------------------
1750 DEF PROCscale
1760 REPEAT
1770 CLS:
     INPUT "CURRENT SCALE - (M)icroamps or (N)anoamps";SC$
1780 UNTIL SC$="M" OR SC$="N"
1790 PRINT
1800 IF SC$="M" THEN
1810 SC1$="Microamps"
1820 ELSE
1830 SC1$="Nanoamps"
1840 ENDIF
1850 REPEAT
1860 PRINT TAB(16);SC1$;" per mm";:
     INPUT SM
1870 UNTIL SM<>0
1880 NApmm=SM:
     IF SC$="M" THEN NApmm=SM*1000
1890 step=250*NApmm
1900 IF 4*step<1000 THEN SC2$=" nanoamps"
1910 IF 4*step>=1000 THEN SC2$="microamps":
     step=step/1000
1920 IF 4*step>=1000 THEN SC2$="milliamps":
     step=step/1000
1930 ENDPROC
1940 :

1950 REM ---------------------------------------------------------------------
1960 DEF PROCholdaxis
1970 CLG
1980 VDU5:
     @%=&00003
1990 MOVE 200,300:
     DRAW1195,300:
     N(1,1)=200:
     N(2,1)=300:
     N(3,1)=1195:
     N(4,1)=300
2000 B=0
2010 DS=995/TS
2020 FOR A=200 TO 1195 STEP DS
2030 MOVEA,295:
     DRAWA,305
2040 N(1,B+2)=A
2050 N(2,B+2)=295
2060 N(3,B+2)=A
2070 N(4,B+2)=305
2080 @%=&01020104
2090 MOVEA-18,282:
     :
     PRINTB:
     T$(B+1)=STR$(B)
2100 T(1,B+1)=A-4*textwidth/2:
     T(2,B+1)=280-textheight
2110 B=B+1
2120 NEXT
2130 B=B+1:
     NT=B
2140 MOVE590,220:
     T$(B)="TIME (MINUTES)":
     PRINTT$(B):
     T(1,B)=590:
     T(2,B)=220
2150 TT=B
2160 @%=&020103
2170 CL$(0)=TYPE$+": HOLD VOLTAGE ="+STR$(HV)+" volt(s)"
2180 PROCscaledraw2
2190 VDU4
2200 ENDPROC
2210 :

2220 REM ---------------------------------------------------------------------
2230 DEF PROCread
2240 B%=0:
     C%=0:
     pread=1:
     SET=-1
2250 CLS:
     PRINT"Press 'ESC' to halt"
2260 LOCAL ERROR
2270 ON ERROR LOCAL PROCerrorlocal
2280 REPEAT
2290 IF SET <> -1 THEN 2430
2300 IF B%<>0 THEN FINISHTIME=FINISHTIME+interval
2310 VAR1%=1000
2320 store%!12=VAR1%
2330 CALL code%
2340 Y%=store%!4
2350 Y=Y%/(VAR1%*4096)
2360 D(0,B%)=((TIME/100)*995/(TS*60)):
     D(1,B%)=(Y*700)+300
2370 PROCscan
2380 B%=B%+1
2390 IF B%<10001 THEN 2410
```

```
2400 *FX 125
2410 REPEAT
2420 UNTIL TIME>FINISHTIME
2430 UNTIL SET <> -1
2440 B%=B%-1
2450 RESTORE ERROR
2460 SF%(0,0)=C%:
     SF%(0,1)=B%
2470 ENDPROC
2480 :

2490 REM ---------------------------------------------------------------------
2500 DEF PROCscan
2510 GCOL0:
     IF B%>199 THEN C%=B%-199
2520 FOR D%=C% TO B%
2530 IF B%<=199 THEN 2580
2540 MOVE D(0,D%-1)-D(0,C%-1)+200,D(1,D%-1)
2550 GCOL3:
     DRAWD(0,D%)-D(0,C%-1)+200,D(1,D%)
2560 GCOL0:
     MOVE D(0,D%-1)-D(0,C%)+200,D(1,D%-1)
2570 IF D%=C%THEN GOTO 2600 ELSE GOTO 2590
2580 IF D%=C% THEN MOVED(0,D%)-D(0,C%)+200,D(1,D%):
     GOTO2600
2590 DRAWD(0,D%)-D(0,C%)+200,D(1,D%)
2600 NEXT D%
2610 ENDPROC
2620 :

2630 REM ---------------------------------------------------------------------
2640 DEF PROCholdselect
2650 A1%=1:
     E%=SF%(0,0):
     F%=SF%(0,1):
     E1%=E%:
     F1%=F%
2660 Repeat%=TRUE
2670 REPEAT
2680 CLS:
     PRINT"Use numeric pad keys to scan - Press 'S' to take SNAPSHOT"
2690 PRINT"          Press 'E' to exit and save data"
2700 IK$=GET$:
     IK=ASC(IK$)
2710 CASE IK OF
2720 WHEN 55:
     ofset%=-50:
     PROCredraw
2730 WHEN 57:
     ofset%=50:
     PROCredraw
2740 WHEN 52:
     ofset%=-10:
     PROCredraw
2750 WHEN 54:
     ofset%=10:
     PROCredraw
2760 WHEN 49:
     ofset%=-1:
     PROCredraw
2770 WHEN 51:
     ofset%=1:
     PROCredraw
2780 WHEN 83,69:
     Repeat%=FALSE
2790 ENDCASE
2800 UNTIL Repeat%=FALSE
2810 IF IK<>83 THEN ENDPROC
2820 SF%(A1%,0)=E1%:
     SF%(A1%,1)=F1%:
     A1%=A1%+1
2830 IF A1%=11 THEN ENDPROC ELSE 2660
2840 ENDPROC
2850 :

2860 REM ---------------------------------------------------------------------
2870 DEF PROCredraw
2880 E1%=E1%+ofset%:
     IF E1%<0 THEN E1%=0
2890 IF E1%>F% THEN E1%=F%
2900 F1%=E1%+199:
     IF F1%>F% THEN F1%=F%
2910 IF F1%<0 THEN F1%=0
2920 PROCholdaxis
2930 FOR D%=E1% TO F1%
2940 IF D%=E1% THEN MOVE 200,D(1,D%):
     GOTO2960
2950 DRAWD(0,D%)-D(0,E1%)+200,D(1,D%)
2960 NEXTD%
2970 GCOL1:
     FOR A%=1TO 10:
     IF SF%(A%,1)=0 THEN A%=10:
     GOTO 3020
2980 FOR D%=SF%(A%,0) TO SF%(A%,1)
2990 IF D%=SF%(A%,0) THEN MOVE200,D(1,D%):
     GOTO3010
3000 DRAWD(0,D%)-D(0,SF%(A%,0))+200,D(1,D%)
3010 NEXTD%
3020 NEXT A%:
     GCOL0
3030 ENDPROC
3040 :

3050 REM ---------------------------------------------------------------------
3060 DEF PROCsweepinput
3070 REPEAT
3080 CLS
3090 IV=0:
     INPUT "INITIAL VOLTAGE";IV:
     PRINT
3100 FV=0:
     INPUT "FINAL VOLTAGE";FV:
```

```
    PRINT
3110 UNTIL ABS(IV)<=1 AND ABS(FV)<=1
3120 REPEAT
3130 INPUT "SWEEP RATE (mV per second)";MV%:
    PRINT
3140 UNTIL MV%<>0
3150 CL$(2)="SWEEP RATE(S): "+STR$(MV%)
3160 ENDPROC
3170 :

3180 REM ------------------------------------------------------------------------
3190 DEF PROCsweepaxis
3200 CLG:
    VDU5
3210 fv1=FV*10:
    iv1=IV*10
3220 dr=SGN(FV-IV):
    nd=ABS(FV-IV)*10
3230 nr=dr
3240 IF nd >10 THEN nr=dr*2
3250 IF nd <5 THEN nr=dr/2
3260 ne=ABS(nr)
3270 ios=(iv1*dr/ne)-(dr*INT(iv1*dr*dr/ne))
3280 fos=(fv1*dr/ne)-(dr*INT(fv1*dr*dr/ne))
3290 IF ios>.001 THEN iv1=iv1-(ios*dr*ne)
3300 IF ABS(fos)>.001 THEN fv1=fv1+(dr*ne)-(fos*dr*ne)
3310 IF ABS(nr)<.6 THEN
3320 @%=&01020000+&0205
3330 ELSE
3340 @%=&01020000+&0104
3350 ENDIF
3360 DS=1000*nr/(fv1-iv1)
3370 MOVE 200,300:
    DRAW1200,300:
    N(1,1)=200:
    N(2,1)=300:
    N(3,1)=1200:
    N(4,1)=300
3380 B=0
3390 FOR A=iv1 TO fv1 STEP nr
3400 N(1,B+2)=200+(B*DS):
    N(2,B+2)=295:
    N(3,B+2)=200+(B*DS):
    N(4,B+2)=305
3410 MOVE N(1,B+2),N(2,B+2):
    DRAW N(3,B+2),N(4,B+2)
3420 MOVE N(1,B+2)-18,282:
    PRINT A/10
3430 T(1,B+1)=N(1,B+2)-4*textwidth/2:
    T(2,B+1)=280-textheight:
    T$(B+1)=STR$(A/10)
3440 B=B+1
3450 NEXT
3460 B=B+1:
    NT=B
3470 @%=&01020205
3480 MOVE620,220:
    T$(B)="VOLTAGE":
    PRINTT$(B):
    T(1,B)=620:
    T(2,B)=220
3490 MOVE200,220:
    PRINT"INIT. VOLT.=";STR$(IV);" volts"
3500 MOVE900,220:
    PRINT"FINAL VOLT.=";STR$(FV);" volts"
3510 CL$(0)=TYPE$
3520 CL$(1)="INITIAL VOLT.= "+STR$(IV)+" volts   FINAL VOLT.= "+STR$(FV)+" volts"
3530 @%=&020103
3540 PROCscaledraw2
3550 TT=B:
    VDU4
3560 ENDPROC
3570 :

3580 REM ------------------------------------------------------------------------
3590 DEF PROCscaledraw2
3600 MOVE 200,300:
    DRAW 200,1000
3610 M1%(1,1)=200
3620 M1%(2,1)=300
3630 M1%(3,1)=200
3640 M1%(4,1)=1000
3650 FOR A%=0TO20
3660 B%=A%*35+300
3670 MOVE 195,B%:
    DRAW 205,B%
3680 M1%(1,A%+2)=195
3690 M1%(2,A%+2)=B%
3700 M1%(3,A%+2)=205
3710 M1%(4,A%+2)=B%
3720 NEXT A%
3730 MOVE 200,650:
    DRAW 1200,650
3740 M1%(1,23)=200
3750 M1%(2,23)=650
3760 M1%(3,23)=1200
3770 M1%(4,23)=650
3780 FOR A%=-5 TO 5
3790 B%=A%*70+650
3800 MOVE 80,B%+8
3810 t$=STR$(A%*step*-1):
    NUL$="        ":
    gap$=LEFT$(NUL$,(6-LEN(t$)))
3820 T1$(A%+6)=gap$+t$
3830 PRINT T1$(A%+6):
    T1(1,A%+6)=68:
    T1(2,A%+6)=B%-(textheight/2)
3840 NEXT A%
3850 MOVE 5,662:
    PRINT SC2$
3860 T2(1)=50:
    T2(2)=506:
```

```
     T2(3)=50:
     T2(4)=810
3870 ENDPROC
3880 :

3890 REM ---------------------------------------------------------------------
3900 DEF PROCread2
3910 SET=-1:
     B%=m%(m%)+1:
     m%=m%+1
3920 pread=2:
     CLS:
     PRINT"Press 'ESC' to halt"
3930 GCOL0:
     S1%=10:
     VAR%=ABS(55642/(MV%/ABS(FV-IV))-42)
3940 IF FS$="C" THEN VAR%=VAR%/2
3950 Y=0:
     X=0:
     store%!12=VAR%
3960 LOCAL ERROR
3970 ON ERROR LOCAL PROCerrorlocal
3980 TIME=0
3990 REPEAT
4000 IF SET <> -1 THEN 4150
4010 CALL code%
4020 X%=!store%:
     Y%=store%!4:
     S%=store%!8
4030 X=((X%/VAR%)-2048)/2048:
     Y=Y%/(4096*VAR%)
4040 D(0,B%)=200+(((10*X-iv1)*1000)/(fv1-iv1))
4050 D(1,B%)=(Y*700)+300
4060 IF TIME<10 THEN4100
4070 IF S%=6 THEN 4140
4080 IF (S1%-S%)*dr>1.99 AND (S1%-S%)*dr<2.01 THEN m%(m%)=B%-1:
     m%=m%+1:
     jump%=0
4090 S1%=S%
4100 IF jump%=0 THEN MOVED(0,B%),D(1,B%):
     jump%=1:
     GOTO4120
4110 DRAWD(0,B%),D(1,B%)
4120 B%=B%+1
4130 IF B%<30000 THEN 4150
4140 *FX 125
4150 UNTIL SET <> -1
4160 m%(m%)=B%-1
4170 RESTORE ERROR
4180 ENDPROC
4190 :

4200 REM ---------------------------------------------------------------------
4210 DEF PROCredraw2
4220 E%=m%(a%-1)+1
4230 FOR D%=E% TO m%(a%)
4240 IF D%=E% THEN MOVE D(0,D%),D(1,D%):
     GOTO 4260
4250 DRAW D(0,D%),D(1,D%)
4260 NEXT D%
4270 ENDPROC
4280 :

4290 REM ---------------------------------------------------------------------
4300 DEF PROCpointer_reset
4310 m%=0:
     m%(m%)=0
4320 ENDPROC
4330 :

4340 REM ---------------------------------------------------------------------
4350 DEF PROCsure
4360 REPEAT
4370 CLS:
     YN$=""
4380 INPUT"Are you sure Y or N - PREVIOUS GRAPH(S) WILL BE ERASED!";YN$
4390 UNTIL YN$="Y" OR YN$="N"
4400 ENDPROC
4410 :

4420 REM ---------------------------------------------------------------------
4430 DEF PROCerase
4440 REPEAT
4450 CLS:
     ER$=""
4460 INPUT"Leave graphs (U)nchanged, erase (L)ast graph or erase (A)ll graphs";ER$
4470 CASE ER$ OF
4480 WHEN "A"
4490 PROCsure
4500 IF YN$="Y" THEN
4510 PROCpointer_reset
4520 ELSE ER$=""
4530 ENDIF
4540 WHEN "L"
4550 m%=m%-1:
     IF m%<0 THEN m%=0
4560 ENDCASE
4570 UNTIL ER$="U" OR ER$="L" OR ER$="A"
4580 ENDPROC
4590 :

4600 REM ---------------------------------------------------------------------
4610 DEF PROCinterupt
4620 SET=-1:
     Z$=""
4630 Repeat%=TRUE
4640 REPEAT
4650 CLS:
     PRINT"PRESS 'R' TO RESET - ";
4660 IF (FS$="L" OR FS$="C") AND pread=1 THEN
4670 PRINT"Press 'S' to display sweep axis or 'X' to exit program ";
4680 GOTO 4720
```

```
4690 ENDIF
4700 PRINT""
4710 PRINT"PRESS 'E' TO EXIT AND SAVE DATA - PRESS 'X' TO EXIT PROGRAM ";
4720 CASE GET$ OF
4730 WHEN "R","r"
4740 Z$="R":
     Repeat%=FALSE:
     PRINT Z$;:
     INPUT"" SILLY$
4750 WHEN "S","s"
4760 Z$="S":
     Repeat%=FALSE:
     PRINT Z$;:
     INPUT"" SILLY$
4770 IF FS$="F" OR pread=2 THEN Repeat%=TRUE
4780 WHEN "E","e"
4790 Z$="E":
     Repeat%=FALSE:
     PRINT Z$;:
     INPUT"" SILLY$
4800 IF FS$<>"F" AND pread=1 THEN Repeat%=TRUE
4810 WHEN "X","x"
4820 Z$="X":
     Repeat%=FALSE
4830 ENDCASE
4840 UNTIL Repeat%=FALSE
4850 SET=0
4860 IF Z$="X" THEN END
4870 ENDPROC
4880 :

4890 REM ---------------------------------------------------------------------
4900 DEF PROCheader
4910 FOR A%=1 TO 27
4920 READ H$(A%)
4930 NEXT
4940 RESTORE
4950 H$(8)=STR$(taxtheight)
4960 ENDPROC
4970 :

4980 REM ---------------------------------------------------------------------
4990 DEF PROCsave
5000 REPEAT
5010 CLS:
     FILE1$="":
     INPUT"Data file name ";FILE1$
5020 UNTIL FILE1$<>""
5030 REPEAT
5040 CLS:
     to$=""
5050 PRINT "          Insert disc (with NAME='Data') in drive 1"
5060 PRINT "               then press C to continue";:
     to$=GET$
5070 UNTIL to$="C"
5080 LOCAL ERROR
5090 ON ERROR LOCAL PROCerror
5100 FILE$=FILE1$
5110 *MOUNT 1
5120 OSCLI("SPOOL "+FILE$)
5130 *SPOOL
5140 OSCLI("DELETE "+FILE$)
5150 RESTORE ERROR
5160 CL$(4)="FILENAME: "+FILE$+"    DATE: "+TIME$:
     PROCcomment
5170 channelout= OPENOUT FILE$
5180 PRINT# channelout, TYPE$
5190 PRINT# channelout, FS$
5200 PRINT# channelout, T$
5210 PRINT# channelout, HV
5220 PRINT# channelout, IV
5230 PRINT# channelout, FV
5240 PRINT# channelout, MV%
5250 PRINT# channelout, NApmm
5260 PRINT# channelout, SC2$
5270 PRINT# channelout, step
5280 FOR count%=0 TO 4
5290 PRINT# channelout, CL$(count%)
5300 NEXT
5310 FOR count%=0 TO 10
5320 PRINT# channelout, SF%(count%,0)
5330 PRINT# channelout, SF%(count%,1)
5340 NEXT
5350 FOR count%= 1 TO 100
5360 IF m%(count%)= 0 THEN
5370 countstore%=count%:
     count%=100
5380 PRINT# channelout, countstore%
5390 ENDIF
5400 NEXT
5410 FOR count%= 0 TO countstore%
5420 PRINT# channelout, m%(count%)
5430 NEXT
5440 PRINT# channelout, m%
5450 count%=0:
     flag%=0
5460 REPEAT
5470 PRINT# channelout, D(0,count%)
5480 PRINT# channelout, D(1,count%)
5490 IF D(0,count%)=0 AND D(1,count%)=0 THEN flag%=1
5500 count%=count%+1
5510 UNTIL flag%=1 OR count%=30001
5520 CLOSE# channelout
5530 *MOUNT 0
5540 ENDPROC
5550 :

5560 REM ---------------------------------------------------------------------
5570 DEFPROCerror
5580 CASE ERR OF
5590 WHEN 17
5600 END
```

```
5610 WHEN 195
5620 REPEAT
5630 CLS:
     PRINT:
     INPUT"  FILE ALREADY EXISTS - REUSE";YN1$
5640 UNTIL YN1$="Y" OR YN1$="N"
5650 IF YN1$="N" THEN 5690
5660 OSCLI("ACCESS "+FILE$)
5670 OSCLI("DELETE "+FILE$)
5680 ENDPROC
5690 REPEAT
5700 FILE1$=FILE$
5710 CLS:
     INPUT"New data file name ";FILE$
5720 UNTIL FILE$<>"" AND FILE$<>FILE1$
5730 FILE1$=""
5740 WHEN 201
5750 CLS:
     PRINT"WRITE PROTECT TAB IS ON - PLEASE REMOVE"
5760 PRINT"PRESS ANY KEY TO CONTINUE":
     IFGET
5770 WHEN 67798
5780 REPEAT
5790 CLS:
     PRINT"Data file not found - files on disk are:-"
5800 PRINT:
     PRINT"     ";
5810 *INFO ADFS:
     :
     1.$.*
5820 PRINT:
     PRINT"(1) Enter new filename"
5830 PRINT:
     PRINT"(2) Change the disk"
5840 PRINT:
     PRINT:
     INPUT "Please enter your choice: ";selection$
5850 CASE selection$ OF
5860 WHEN "1"
5870 FILE$=FILE1$
5880 REPEAT
5890 PRINT:
     INPUT"New data file name ";FILE1$
5900 UNTIL FILE1$<>"" AND FILE$<>FILE1$
5910 FILE$="":

5920 WHEN "2"
5930 CLS:
     PRINT TAB(10,10);"Press any key when disk has been changed":
     IFGET
5940 *MOUNT 1
5950 ENDCASE
5960 UNTIL selection$="1" OR selection$="2"
5970 OTHERWISE
5980 PRINT
5990 REPORT:
     PRINT " AT LINE";ERL;" (Error No. ";ERR;")";
6000 PRINT" - PRESS ANY KEY TO CONTINUE":
     IFGET
6010 ENDCASE
6020 ENDPROC
6030 :

6040 REM ------------------------------------------------------------------------
6050 DEFPROCerrorlocal
6060 IF ERR<>17 THEN
6070 REPORT:
     PRINT " AT LINE ";ERL;" (Error No. ";ERR;")"
6080 PRINT"PRESS ANY KEY TO CONTINUE"
6090 IFGET
6100 ENDPROC
6110 ENDIF
6120 PROCinterupt
6130 ENDPROC
6140 :

6150 REM ------------------------------------------------------------------------
6160 DEF PROCcode
6170 osbyte%=6
6180 readbyte%=146
6190 DIM code% 400,store% 20
6200 FOR pass%=0 TO 2 STEP2
6210 P%=code%
6220 [
6230 OPT pass%
6240 STMFD R13!,(R0-R12,R14)
6250 ADR R8,store%
6260 LDR R3,[R8,#12]
6270 MOV R0,#readbyte%
6280 .start
6290 MOV R1,#0
6300 SWI osbyte%
6310 ADDAL R4,R4,R2,LSL#8
6320 MOV R1,#1
6330 SWI osbyte%
6340 ADDAL R4,R4,R2
6350 MOV R1,#2
6360 SWI osbyte%
6370 ADDAL R5,R5,R2,LSL#8
6380 MOV R1,#3
6390 SWI osbyte%
6400 ADDAL R5,R5,R2
6410 MOV R1,#5
6420 SWI osbyte%
6430 ADD R7,R7,#1
6440 TEQ R3,R7
6450 BNE start
6460 MOV R1,#6
6470 SWI osbyte%
6480 ADDAL R6,R6,R2
6490 ADR R8,store%
6500 STMIA R8, {R4,R5,R6}
```

```
6510 LDMFD R13!,(R0-R12,PC)
6520 ]
6530 NEXT pass%
6540 ENDPROC
6550 :

6560 REM ------------------------------------------------------------------------
6570 DEF PROCcomment
6580 IF CL$(2)<>"" AND RIGHT$(CL$(2),12)<>" mV per sec." THEN CL$(2)=CL$(2)+" mV per sec."
6590 FOR A%=0 TO 4
6600 CLS
6610 PRINT"ENTER 'D' to delete comment, or 'RETURN' to leave comment unchanged"
6620 PRINT
6630 PRINT "Comment line ";A%+1;": ";CL$(A%):
     CL$="":
     INPUT"COMMENT: ";CL$
6640 IF CL$="" THEN CL$=CL$(A%)
6650 IF CL$="D" THEN CL$=""
6660 IF LEN(CL$)>69 THEN 6600
6670 CL$(A%)=CL$
6680 NEXTA%
6690 CLS:
     YN$="":
     REPEAT:
     INPUT"OK. Y or N";YN$:
     UNTIL YN$="Y" OR YN$="N"
6700 IF YN$="N" THEN 6590
6710 ENDPROC
6720 :

6730 REM ------------------------------------------------------------------------
6740 DEFPROCedit
6750 CLS:
     CLG:
     MODE8
6760 COLOUR 131:
     COLOUR 1:
     GCOL 131:
     GCOL 0
6770 REPEAT
6780 CLS:
     FILE1$="":
     INPUT TAB(5,9);"File name of data to be edited";FILE1$
6790 UNTIL FILE1$<>""
6800 REPEAT
6810 CLS:
     tc$=""
6820 PRINT TAB(5,9);"Insert disc in drive 1 then press C to continue";:
     tc$=GET$
6830 UNTIL tc$="C"
6840 LOCAL ERROR
6850 ON ERROR LOCAL PROCerror
6860 *MOUNT 1
6870 PRINT:
     PRINT
6880 OSCLI("INFO "+FILE1$)
6890 PRINT
6900 channelin= OPENIN FILE1$
6910 INPUT# channelin, TYPE$
6920 INPUT# channelin, FS$
6930 INPUT# channelin, TS
6940 INPUT# channelin, HV
6950 INPUT# channelin, IV
6960 INPUT# channelin, FV
6970 INPUT# channelin, MV%
6980 INPUT# channelin, NApmm
6990 INPUT# channelin, SC2$
7000 INPUT# channelin, step
7010 FOR count%=0 TO 4
7020 INPUT# channelin, CL$(count%)
7030 NEXT
7040 FOR count%=0 TO 10
7050 INPUT# channelin, SF%(count%,0)
7060 INPUT# channelin, SF%(count%,1)
7070 NEXT
7080 INPUT# channelin, countstore%
7090 FOR count%= 0 TO countstore%
7100 INPUT# channelin, m%(count%)
7110 NEXT
7120 INPUT# channelin, m%
7130 count%=0:
     flag%=0
7140 REPEAT
7150 INPUT# channelin, D(0,count%)
7160 INPUT# channelin, D(1,count%)
7170 count%=count%+1
7180 UNTIL EOF# channelin
7190 CLOSE# channelin
7200 OSCLI("ACCESS "+FILE1$+" LR")
7210 RESTORE ERROR
7220 *MOUNT 0
7230 :

7240 REM          OUTPUT FORMAT MENU
7250 REPEAT
7260 CLS
7270 PRINT TAB(10,5);"(1) Produce DESIGNCAD file"
7280 PRINT TAB(10,10);"(2) Produce POSTSCRIPT file"
7290 PRINT TAB(10,15);"(3) Produce ENCAPSUALTED POSTSCRIPT file"
7300 INPUT TAB(15,22);"Please enter your choice: ";output_type$
7310 CASE output_type$ OF
7320 WHEN "1"
7330 PROCoutput_filename
7340 PROCoutput_redraw
7350 PROCdesigncad
7360 WHEN "2"
7370 PROCoutput_filename
7380 PROCoutput_redraw
7390 PROCpostscript_menu("PS")
7400 WHEN "3"
7410 PROCoutput_filename
7420 PROCoutput_redraw
```

```
7430 PROCpostscript_menu("EPS")
7440 ENDCASE
7450 UNTIL output_type$="1" OR output_type$="2" OR output_type$="3"
7460 ENDPROC
7470 :

7480 REM ------------------------------------------------------------------------
7490 DEF PROCoutput_redraw
7500 VDU 28,0,31,79,27
7510 IF FS$="F" THEN
7520 CLG:
     PROCholdaxis
7530 FOR a%=0 TO 10
7540 IF SF%(a%,1)=0 THEN a%=10:
     GOTO 7560
7550 C%=SF%(a%,0):
     B%=SF%(a%,1):
     PROCscan
7560 NEXT a%
7570 ELSE
7580 CLG:
     PROCsweepaxis
7590 FOR a%=1 TO m%
7600 PROCredraw2
7610 NEXT a%
7620 ENDIF
7630 ENDPROC
7640 :

7650 REM ------------------------------------------------------------------------
7660 DEF PROCoutput_filename
7670 Disk_Printer$="D"
7680 CASE output_type$ OF
7690 WHEN "1"
7700 output_filename$=FILE1$+"D"
7710 WHEN "2"
7720 output_filename$=FILE1$+"P"
7730 REPEAT
7740 CLS:
     INPUT TAB(10,5);" Output file to (D)isc or (P)rinter";Disk_Printer$
7750 UNTIL Disk_Printer$="D" OR Disk_Printer$="P"
7760 WHEN "3"
7770 output_filename$=FILE1$+"E"
7780 ENDCASE
7790 IF Disk_Printer$="D" THEN
7800 CLS:
     PRINT:
     PRINT
7810 PRINT" Enter NEW OUTPUT FILENAME, or press 'RETURN' to leave unchanged"
7820 PRINT
7830 PRINT"     FILENAME: ";output_filename$;:
     INPUTedit_filename$
7840 IF edit_filename$<>"" THEN output_filename$=edit_filename$
7850 FILE$=output_filename$
7860 ELSE
7870 FILE$="TPIRCSTSOP" :
      REM unlikely filename (POSTSCRIPT backwards)
7880 ENDIF
7890 LOCAL ERROR
7900 ON ERROR LOCAL PROCerror
7910 IF Disk_Printer$="D" THEN
7920 *MOUNT 1
7930 ELSE
7940 *MOUNT 0
7950 ENDIF
7960 OSCLI("SPOOL "+FILE$)
7970 *SPOOL
7980 OSCLI("DELETE "+FILE$)
7990 RESTORE ERROR
8000 ENDPROC
8010 :

8020 REM ------------------------------------------------------------------------
8030 DEF PROCdesigncad
8040 IF FS$="F" THEN
8050 PROCopenfile(-1)
8060 FOR a%=0 TO 10
8070 IF SF%(a%,1)=0 THEN a%=10:
     GOTO 8150
8080 CLG:
     PROCholdaxis:
     C%=SF%(a%,0):
     B%=SF%(a%,1):
     PROCscan
8090 REPEAT
8100 CLS:
     YN$="":
     INPUT"Do you want to save this screen Y or N";YN$
8110 UNTIL YN$="Y" OR YN$="N"
8120 IF YN$="N" THEN 8150
8130 EL$="1 "+STR$(B%-C%+1)+" 1 0 0 1"
8140 PROCopenfile(0)
8150 NEXT a%
8160 ELSE
8170 PROCopenfile(-1)
8180 FOR a%=1 TO m%
8190 CLG:
     PROCsweepaxis
8200 PROCredraw2
8210 REPEAT
8220 CLS:
     YN$="":
     INPUT"Do you want to save this screen Y or N";YN$
8230 UNTIL YN$="Y" OR YN$="N"
8240 IF YN$="N" THEN 8260
8250 PROCopenfile(1)
8260 NEXT a%
8270 ENDIF
8280 OSCLI("ACCESS "+FILE$+" LR")
8290 *MOUNT 0
8300 ENDPROC
8310 :
```

```
8320 REM ---------------------------------------------------------------------
8330 DEF PROCpostscript_menu(ps_or_eps$)
8340 :

8350 REM          POSTSCRIPT FORMAT MENU
8360 CLS
8370 ps_eps$=ps_or_eps$
8380 CASE FS$ OF
8390 WHEN "F"
8400 REPEAT
8410 REPEAT
8420 FOR a%=0 TO 10
8430 IF SF%(a%,1)=0 THEN
8440 snapshots%=a%-1:
     a%=10
8450 PRINT " Number of snapshots taken = ";snapshots%
8460 ENDIF
8470 NEXT a%
8480 PRINT "    (1) Produce output of all SNAPSHOTS"
8490 PRINT "    (2) Produce output selected SNAPSHOTS"
8500 PRINT "    (3) Exit"
8510 PRINT
8520 INPUT "Please enter your choice: ";psoutput_type$
8530 CASE psoutput_type$ OF
8540 WHEN "1"
8550 PROCpostscript("ALL")
8560 WHEN "2"
8570 PROCpostscript("SELECT")
8580 ENDCASE
8590 UNTIL psoutput_type$="1" OR psoutput_type$="2" OR psoutput_type$="3"
8600 UNTIL psoutput_type$="3"
8610 OTHERWISE
8620 REPEAT
8630 REPEAT
8640 PRINT " Number of sweeps in current file = ";m%
8650 PRINT "   (1) Produce output of all sweeps"
8660 PRINT "   (2) Produce output of several selected sweeps"
8670 PRINT "   (3) Produce output of single sweep (analysis and enlargement possible)"
8680 INPUT "   (4) Exit";psoutput_type$
8690 CASE psoutput_type$ OF
8700 WHEN "1"
8710 PROCpostscript("ALL")
8720 WHEN "2"
8730 PROCpostscript("SELECT")
8740 WHEN "3"
8750 PROCpostscript("SINGLE")
8760 ENDCASE
8770 UNTIL psoutput_type$="1" OR psoutput_type$="2" OR psoutput_type$="3" OR psoutput_type$="4"
8780 UNTIL psoutput_type$="4"
8790 ENDCASE
8800 ENDPROC
8810 :

8820 REM ---------------------------------------------------------------------
8830 DEF PROCpostscript(All_Select_Single$)
8840 IF FS$="F" THEN
8850 PROCopenpsfile(-1)
8860 FOR a%=0 TO 10
8870 IF SF%(a%,1)=0 THEN a%=10:
     GOTO 8970
8880 CLG:
     PROCholdaxis
8890 C%=SF%(a%,0):
     B%=SF%(a%,1)
8900 PROCscan
8910 IF All_Select_Single$="ALL" THEN 8960
8920 REPEAT
8930 CLS:
     YN$="":
     INPUT"Do you want to save this screen Y or N";YN$
8940 UNTIL YN$="Y" OR YN$="N"
8950 IF YN$="N" THEN 8970
8960 PROCopenpsfile(1)
8970 NEXT a%
8980 PROCopenpsfile(2)
8990 OSCLI("ACCESS "+FILE$+" LR")
9000 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
9010 *MOUNT 0
9020 ELSE
9030 CASE All_Select_Single$ OF
9040 WHEN "ALL"
9050 PROCopenpsfile(-1)
9060 FOR a%=1 TO m%
9070 PROCopenpsfile(1)
9080 NEXT a%
9090 PROCopenpsfile(2)
9100 OSCLI("ACCESS "+FILE$+" LR")
9110 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
9120 *MOUNT 0
9130 WHEN "SELECT"
9140 PROCopenpsfile(-1)
9150 FOR a%=1 TO m%
9160 CLG:
     PROCsweepaxis
9170 PROCredraw2
9180 REPEAT
9190 CLS:
     YN$="":
     INPUT"Do you want to save this screen Y or N";YN$
9200 UNTIL YN$="Y" OR YN$="N"
9210 IF YN$="N" THEN 9080
9220 PROCopenpsfile(1)
9230 NEXT a%
9240 PROCopenpsfile(2)
9250 OSCLI("ACCESS "+FILE$+" LR")
9260 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
9270 *MOUNT 0
9280 WHEN "SINGLE"
9290 CLS:
     a%=1
9300 *FX4,1
```

```
9310 REPEAT
9320 CLG
9330 PROCsweepaxis
9340 PROCredraw2
9350 PRINT " This is sweep number ";a%;" out of ";m%;" sweeps":
     PRINT
9360 PRINT " Use cursor keys to select required sweep then press 'RETURN'"
9370 IK$=GET$:
     IK=ASC(IK$)
9380 CASE IK OF
9390 WHEN 136
9400 IF a%>1 THEN
9410 a%=a%-1
9420 ENDIF
9430 WHEN 137
9440 IF a%<m% THEN
9450 a%=a%+1
9460 ENDIF
9470 ENDCASE
9480 UNTIL IK=13
9490 *FX4,0
9500 Selected_sweep%=a%
9510 IF ps_eps$="EPS" THEN
9520 PROCopenpsfile(-1)
9530 PROCopenpsfile(1)
9540 PROCopenpsfile(2)
9550 OSCLI("ACCESS "+FILE$+" LR")
9560 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
9570 *MOUNT 0
9580 ELSE
9590 PROCsingle_analysis
9600 ENDIF
9610 ENDCASE
9620 ENDIF
9630 IF Disk_Printer$="P" THEN
9640 *MOUNT 0
9650 CLS:
     PRINT:
     PRINT"  Printing - Please Wait......."
9660 OSCLI("ACCESS "+FILE$+" WR")
9670 OSCLI("COPY "+FILE$+" PRINTER: -C-VDQ")
9680 ENDIF
9690 ENDPROC
9700 :

9710 REM ---------------------------------------------------------------------
9720 DEF PROCopenfile(v%)
9730 LOCAL ERROR
9740 ON ERROR LOCAL PROCerror
9750 IF v%=-1 THEN
9760 OSCLI("SPOOL "+FILE$)
9770 ELSE
9780 OSCLI("SPOOLON "+FILE$)
9790 ENDIF
9800 PROCdatawrite(v%)
9810 *SPOOL
9820 RESTORE ERROR
9830 ENDPROC
9840 :

9850 REM ---------------------------------------------------------------------
9860 DEFPROCdatawrite(w%)
9870 IF w%<>-1 THEN 10300
9880 FOR A%=1TO27
9890 PRINT H$(A%)
9900 NEXT
9910 PRINT "1 2 1 0 0 0":
     PRINT "0 0":
     PRINT "0 0"
9920 PRINT "1 2 1 0 0 0":
     PRINT "0 1010":
     PRINT "0 1010"
9930 PRINT "1 2 1 0 0 0":
     PRINT "1280 1010":
     PRINT "1280 1010"
9940 PRINT "1 2 1 0 0 0":
     PRINT "1280 0":
     PRINT "1280 0"
9950 PRINT "21 0 0 0 0 0"
9960 PRINT "1 5 1 0 0 1":
     PRINT"280 0":
     PRINT"1120 0"
9970 PRINT"1120 187":
     PRINT"280 187":
     PRINT"280 0"
9980 FOR A%=0 TO 4
9990 PRINT "3 2 16 0 0 1"
10000 PRINT "300 "+STR$(153-(34*A%))
10010 PRINT "1100 "+STR$(153-(34*A%))
10020 PRINT CL$(A%)
10030 NEXT
10040 FOR A%=1 TO NT
10050 PRINT"1 2 1 0 0 1"
10060 PRINT STR$(N(1,A%))+" "+STR$(N(2,A%))
10070 PRINT STR$(N(3,A%))+" "+STR$(N(4,A%))
10080 NEXT A%
10090 @%=&020106
10100 FOR A%=1 TO TT
10110 PRINT"3 1 16 0 0 1"
10120 PRINT STR$(T(1,A%))+" "+STR$(T(2,A%))
10130 PRINT T$(A%)
10140 NEXT
10150 FOR A%=1TO23
10160 PRINT "1 2 1 0 0 1"
10170 PRINT STR$(M1%(1,A%))+" "+STR$(M1%(2,A%))
10180 PRINT STR$(M1%(3,A%))+" "+STR$(M1%(4,A%))
10190 NEXT
10200 FOR A%=1TO11
10210 PRINT "3 1 16 0 0 1"
10220 PRINT STR$(T1(1,A%))+" "+STR$(T1(2,A%))
10230 PRINT T1$(A%)
10240 NEXT
```

```
10250 PRINT "3 2 16 0 0 1"
10260 PRINT STR$(T2(1))+" "+STR$(T2(2))
10270 PRINT STR$(T2(3))+" "+STR$(T2(4))
10280 PRINT "CURRENT ("+SC2$+")"
10290 ENDPROC
10300 IF FS$="F" THEN
10310 PRINT "21 "+STR$(a%+1)+" 0 0 0 0"
10320 PRINT EL$
10330 FOR A%=C% TO B%
10340 PRINT STR$(D(0,A%)-D(0,C%)+200)+" "+STR$(D(1,A%))
10350 NEXT
10360 ELSE
10370 PROCsweepout
10380 ENDIF
10390 ENDPROC
10400 :

10410 REM ------------------------------------------------------------------------
10420 DEF PROCsweepout
10430 PRINT "21 "+STR$(a%)+" 0 0 0 0"
10440 e%=m%(a%-1)+1:
      IF a%>1 THEN e%=e%+1
10450 f%=m%(a%)-e%
10460 IF f%=199 THEN f%=200 ELSE f%=199
10470 FOR b%=e% TO m%(a%) STEP f%
10480 d%=199:
      IF m%(a%)-b%<200 THEN d%=m%(a%)-b%
10490 PRINT "1 "+STR$(d%+1)+" 1 0 0 1"
10500 FOR c%=b% TO b%+d%
10510 PRINT STR$(D(0,c%))+" "+STR$(D(1,c%))
10520 NEXT
10530 NEXT
10540 ENDPROC
10550 :

10560
10570 REM ------------------------------------------------------------------------
10580 DEF PROCopenpsfile(v%)
10590 LOCAL ERROR
10600 ON ERROR LOCAL PROCerror
10610 IF v%<0 THEN
10620 OSCLI("SPOOL "+FILE$)
10630 ELSE
10640 OSCLI("SPOOLON "+FILE$)
10650 ENDIF
10660 IF v%<>2 THEN
10670 PROCpswrite(v%)
10680 ELSE
10690 PRINT "state restore"
10700 IF ps_eps$="PS" THEN
10710 PRINT "showpage"
10720 ELSE
10730 PRINT"^D"
10740 ENDIF
10750 ENDIF
10760 *SPOOL
10770 RESTORE ERROR
10780 ENDPROC
10790 :

10800 REM ------------------------------------------------------------------------
10810 DEF PROCpswrite(w%)
10820 REM  - Postscript file writing routine -
10830 REM  - if w%<0 = Write Header as follows
10840 REM  -    w%=-1 = Standadrd sized plain drawing
10850 REM  -    w%=-2 = Enlarged drawing with comment box
10860 REM  -    w%=-3 = Standard size drawing with peak analysis
10870 REM  -    w%=-4 = Differentiated curve - enlarged with comment box
10880 REM  -    w%=-5 = Differentiated curve - Standard size drawing
10890 REM  - if w%>0 = Write Curve Data
10900 REM
10910 IF w%>0 THEN 13160
10920 IF ps_eps$="EPS" THEN
10930 PRINT"%!PS-Adobe-2.0 EPSF-1.2"
10940 PRINT"%%Creator: Cylic voltammetry program"
10950 PRINT"%%For: WordPerfect input"
10960 PRINT"%%Title: ";FILE$
10970 PRINT"%%CreationDate: ";TIME$
10980 PRINT"%%BoundingBox: 0 0 712 792"
10990 PRINT"%%DocumentFonts: Times-Roman"
11000 PRINT"%%EndComments"
11010 ELSE
11020 PRINT "%!";
      PRINT "%%EndComments"
11030 ENDIF
11040 PRINT
11050 PRINT "%-----------------Prologue---------------"
11060 PRINT "/_rm"
11070 PRINT "    {rmoveto} bind def"
11080 PRINT "/_rl"
11090 PRINT "    {rlineto} bind def"
11100 PRINT "/_s"
11110 PRINT "    {show} bind def"
11120 PRINT
11130 IF w%=-3 THEN
11140 PRINT "%---------Peak Current Text procs-------"
11150 PRINT "/ispo"
11160 PRINT "    {8 12 _rm _lf setfont (\(I) _s _sf setfont (sp) _s"
11170 PRINT "     _lf setfont (\)) _s 0 -5 _rm _sf setfont (o) _s"
11180 PRINT "     -8 -7 _rm} def"
11190 PRINT
11200 PRINT "/ipco"
11210 PRINT "    {8 12 _rm _lf setfont (\(I) _s _sf setfont (pc) _s"
11220 PRINT "     _lf setfont (\)) _s 0 -5 _rm _sf setfont (o) _s"
11230 PRINT "     -8 -7 _rm} def"
11240 PRINT
11250 PRINT "/ipao"
11260 PRINT "    {8 12 _rm _lf setfont (\(I) _s _sf setfont (pa) _s"
11270 PRINT "     _lf setfont (\)) _s 0 -5 _rm _sf setfont (o) _s"
11280 PRINT "     -8 -7 _rm} def"
11290 PRINT
11300 PRINT "/ipc"
```

```
11310 PRINT "     (8 12 _rm _lf setfont (  I) _s _sf setfont (pc  ) _s"
11320 PRINT "      -8 -12 _rm) def"
11330 PRINT
11340 PRINT "/ipa"
11350 PRINT "     (8 12 _rm _lf setfont (  I) _s _sf setfont (pa  ) _s"
11360 PRINT "      -8 -12 _rm) def"
11370 PRINT
11380 ENDIF
11390 PRINT "%%EndProlog"
11400 PRINT
11410 PRINT "%-----------------SCRIPT----------------"
11420 PRINT "/state save def"
11430 PRINT
11440 PRINT "%---------------Define Fonts------------"
11450 PRINT "%large font lf"
11460 PRINT "/_lf"
11470 PRINT "     /Times-Roman findfont 24 scalefont def"
11480 PRINT
11490 PRINT "%medium font mf"
11500 PRINT "/_mf"
11510 PRINT "     /Times-Roman findfont 20 scalefont def"
11520 PRINT
11530 PRINT "%small font sf"
11540 PRINT "/_sf"
11550 PRINT "     /Times-Roman findfont 16 scalefont def"
11560 PRINT
11570 PRINT "%---------Move Origin and Scale---------"
11580 IF w%=-2 OR w%=-4 THEN
11590 PRINT "%Enlarged Drawing - Portrait orientation"
11600 PRINT "1.5 .566929 mul dup scale"
11610 PRINT Xoffset%*-1;" ";Yoffset%*-1;" translate"
11620 PRINT
11630 ELSE
11640 PRINT "%Normal Sized Drawing Landscape orientation"
11650 PRINT "20 792 translate"
11660 PRINT "-90 rotate"
11670 PRINT ".566929 dup scale"
11680 PRINT
11690 ENDIF
11700 PRINT "%---------Draw Main Comment Box---------"
11710 PRINT "_sf setfont"
11720 PRINT "330 0 moveto":
      PRINT"1070 0 lineto"
11730 PRINT "1070 132 lineto":
      PRINT"330 132 lineto":
      PRINT"closepath"
11740 PRINT "stroke"
11750 FOR A%=0 TO 4
11760 PRINT "350 "+STR$(108-(24*A%))+" moveto"
11770 PRINT "("+CL$(A%)+") _s"
11780 PRINT
11790 NEXT
11800 IF w%<>-2 AND w%<>-4 THEN
11810 PRINT "%----Translation before drawing Axis----"
11820 PRINT "0 -40 translate"
11830 PRINT
11840 ENDIF
11850 IF w%<-1 AND w%>=-4 THEN
11860 PRINT "%------------Draw Comment Box------------"
11870 PRINT STR$(CommentBoxX%)+" "+STR$(CommentBoxY%)+" moveto"
11880 PRINT "%store box position in postscript variables Xpos and Ypos"
11890 PRINT "currentpoint"
11900 PRINT "/Ypos exch def"
11910 PRINT "/Xpos exch def"
11920 PRINT STR$(CommentBoxSizeX%)+" 0 _rl"
11930 PRINT "0 "+STR$(CommentBoxSizeY%)+" _rl"
11940 PRINT "-"+STR$(CommentBoxSizeX%)+" 0 _rl"
11950 PRINT "closepath"
11960 PRINT
11970 CASE w% OF
11980 WHEN -2,-4
11990 PRINT "%--Fill Comment Box (enlargement)-------"
12000 PRINT "Xpos Ypos moveto"
12010 PRINT "6 22 _rm"
12020 PRINT "("+CommentLine1$+") _s"
12030 PRINT "Xpos Ypos moveto"
12040 PRINT "6 6 _rm"
12050 PRINT "("+CommentLine2$+") _s"
12060 PRINT
12070 WHEN -3
12080 @%=&0102030A
12090 PRINT "%-----Fill Comment Box (analysis)-------"
12100 PRINT "Xpos Ypos moveto"
12110 PRINT "ipc 8 12 _rm _mf setfont ( = "+STR$(Ipc)+", ) _s 0 -12 _rm"
12120 PRINT "ipa 8 12 _rm _mf setfont ( = "+STR$(Ipa)+", ) _s 0 -12 _rm"
12130 PRINT "ispo 8 12 _rm _mf setfont ( = "+STR$(Ispo)+" ) _s 0 -12 _rm"
12140 PRINT "Xpos Ypos moveto"
12150 PRINT "0 70 _rm"
12160 IF dr<0 THEN PRINT "ipa" ELSE PRINT "ipc"
12170 PRINT "40 0 _rm"
12180 IF dr<0 THEN PRINT "ipao" ELSE PRINT "ipco"
12190 PRINT "58 12 _rm _mf setfont (0.485) _s"
12200 PRINT "10 10 _rm 10 -10 _rl -10 0 _rm 10 10 _rl 0 -10 _rm"
12210 PRINT "2 -12 _rm ispo stroke"
12220 PRINT "Xpos Ypos moveto"
12230 PRINT "0 40 _rm"
12240 IF dr<0 THEN PRINT "ipc" ELSE PRINT "ipa"
12250 PRINT "40 0 _rm"
12260 IF dr<0 THEN PRINT "ipc" ELSE PRINT "ipa"
12270 PRINT "95 0 _rm"
12280 IF dr<0 THEN PRINT "ipc" ELSE PRINT "ipa"
12290 PRINT "Xpos Ypos moveto"
12300 PRINT "15 72 _rm 30 0 _rl 15 3 _rm 14 0 _rl -14 -6 _rm 14 0 _rl"
12310 PRINT "15 3 _rm 50 0 _rl 15 0 _rm 14 0 _rl -7 -7 _rm 0 14 _rl 7 -7 _rm"
12320 PRINT "15 0 _rm 130 0 _rl 15 0 _rm 14 0 _rl -7 -7 _rm 0 14 _rl 7 -7 _rm"
12330 PRINT "10 -7 _rm _mf setfont (0.086) _s 10 7 _rm"
12340 PRINT "10 3 _rm 14 0 _rl -14 -6 _rm 14 0 _rl 10 -4 _rm"
12350 PRINT "("+STR$(Analysis_Result)+") _s stroke"
12360 PRINT
12370 ENDCASE
12380 ENDIF
12390 PRINT "%--------------Draw Axis----------------"
```

```
12400 PRINT "_lf setfont"
12410 FOR A%=1 TO NT
12420 PRINT STR$(N(1,A%))+" "+STR$(N(2,A%))+" moveto"
12430 PRINT STR$(N(3,A%))+" "+STR$(N(4,A%))+" lineto"
12440 PRINT "stroke"
12450 NEXT A%
12460 @%=&020106
12470 FOR A%=1 TO TT
12480 PRINT STR$(T(1,A%)+16)+" "+STR$(T(2,A%))+" moveto"
12490 PRINT "("+T$(A%)+") _s"
12500 NEXT
12510 FOR A%=1TO23
12520 PRINT STR$(M1%(1,A%))+" "+STR$(M1%(2,A%))+" moveto"
12530 PRINT STR$(M1%(3,A%))+" "+STR$(M1%(4,A%))+" lineto"
12540 PRINT "stroke"
12550 NEXT
12560 FOR A%=1TO11
12570 PRINT STR$(T1(1,A%)+62)+" "+STR$(T1(2,A%))+" moveto"
12580 PRINT "("+T1$(A%)+") _s"
12590 NEXT
12600 PRINT STR$(T2(1)+70)+" "+STR$(T2(2))+" translate"
12610 PRINT "90 rotate"
12620 PRINT "0 0 moveto"
12630 PRINT "(Current ("+SC2$+")) _s"
12640 PRINT "-90 rotate"
12650 PRINT "-"+STR$(T2(1)+70)+" -"+STR$(T2(2))+" translate"
12660 PRINT "0.5 setlinewidth"
12670 PRINT
12680 IF w%=-3 THEN
12690 PRINT "%------------Analysis Legend------------"
12700 PRINT STR$(D(0,Yminpos))+" "+STR$(D(1,Yminpos))+" moveto"
12710 PRINT STR$(D(0,Yminpos))+" 650 lineto stroke"
12720 PRINT "gsave"
12730 PRINT STR$(D(0,Yminpos))+" "+STR$((D(1,Yminpos)+650)/2)+" translate"
12740 PRINT "0 0 moveto"
12750 PRINT "0.7 0.7 scale"
12760 IF dr<0 THEN PRINT "ipc" ELSE PRINT "ipco"
12770 PRINT "grestore"
12780 PRINT STR$(D(0,Ymaxpos))+" "+STR$(D(1,Ymaxpos))+" moveto"
12790 PRINT STR$(D(0,Ymaxpos))+" 650 lineto stroke"
12800 PRINT "gsave"
12810 PRINT STR$(D(0,Ymaxpos))+" "+STR$((D(1,Ymaxpos)+650)/2)+" translate"
12820 PRINT "0 0 moveto"
12830 PRINT "0.7 0.7 scale"
12840 IF dr<0 THEN PRINT "ipao" ELSE PRINT "ipa"
12850 PRINT "grestore"
12860 PRINT STR$(D(0,Xmaxpos))+" "+STR$(D(1,Xmaxpos))+" moveto"
12870 PRINT STR$(D(0,Xmaxpos))+" 650 lineto stroke"
12880 PRINT "gsave"
12890 PRINT STR$(D(0,Xmaxpos))+" "+STR$((D(1,Xmaxpos)+650)/2)+" translate"
12900 PRINT "0 0 moveto"
12910 PRINT "0.7 0.7 scale"
12920 PRINT "ispo"
12930 PRINT "grestore"
12940 PRINT
12950 ENDIF
12960 IF w%=-4 OR w%=-5 THEN
12970 FOR count%=fss% TO fse%-step% STEP 1
12980 IF count%=fss% THEN
12990 PRINT STR$(F(0,count%-fss%))+" "+STR$(F(1,count%-fss%)*Scale_factor+650)+" moveto"
13000 ELSE
13010 PRINT STR$(F(0,count%-fss%))+" "+STR$(F(1,count%-fss%)*Scale_factor+650)+" lineto"
13020 ENDIF
13030 NEXT
13040 PRINT "stroke"
13050 FOR count%=rss% TO rse%-step% STEP 1
13060 IF count%=rss% THEN
13070 PRINT STR$(R(0,count%-rss%))+" "+STR$(R(1,count%-rss%)*Scale_factor+650)+" moveto"
13080 ELSE
13090 PRINT STR$(R(0,count%-rss%))+" "+STR$(R(1,count%-rss%)*Scale_factor+650)+" lineto"
13100 ENDIF
13110 NEXT
13120 PRINT "stroke"
13130 PRINT "[10] 5 setdash"
13140 ENDIF
13150 ENDPROC
13160 IF FS$="F" THEN
13170 FOR A%=C% TO B%
13180 IF A%=C% THEN
13190 PRINT STR$(D(0,A%)-D(0,C%)+200)+" "+STR$(D(1,A%))+" moveto"
13200 ELSE
13210 PRINT STR$(D(0,A%)-D(0,C%)+200)+" "+STR$(D(1,A%))+" lineto"
13220 ENDIF
13230 NEXT
13240 PRINT "stroke"
13250 ELSE
13260 PROCps_sweepout
13270 ENDIF
13280 ENDPROC
13290 :

13300
13310 REM ------------------------------------------------------------------------
13320 DEF PROCps_sweepout
13330 e%=m%(a%-1)+1
13340 FOR b%=e% TO m%(a%)
13350 IF b%=e% THEN
13360 PRINT STR$(D(0,b%))+" "+STR$(D(1,b%))+" moveto"
13370 ELSE
13380 PRINT STR$(D(0,b%))+" "+STR$(D(1,b%))+" lineto"
13390 ENDIF
13400 NEXT
13410 PRINT "stroke"
13420 ENDPROC
13430 :

13440 REM ------------------------------------------------------------------------
13450 DEF PROCsingle_analysis
13460 :

13470 REM          ANALYSIS FORMAT MENU
13480 MODE8:
```

```
      COLOUR 131:
      COLOUR 1:
      GCOL 131:
      GCOL 0
13490 REPEAT
13500 CLS
13510 PRINT TAB(10,5);"(1) Print"
13520 PRINT TAB(10,8);"(2) Print Enlargement"
13530 PRINT TAB(10,11);"(3) Calculate Peak Ratios"
13540 PRINT TAB(10,14);"(4) Print differentiated curve"
13550 PRINT TAB(10,17);"(5) EXIT"
13560 INPUT TAB(15,22);"Please enter your choice: ";analysis_type$
13570 CASE analysis_type$ OF
13580 WHEN "1"
13590 MODE8:
      VDU28,0,31,79,27
13600 COLOUR 131:
      COLOUR 1:
      GCOL 131:
      GCOL 0
13610 CLG:
      PROCsweepaxis
13620 PROCredraw2
13630 PROCopenpsfile(-1)
13640 PROCopenpsfile(1)
13650 PROCopenpsfile(2)
13660 OSCLI("ACCESS "+FILE$+" LR")
13670 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
13680 *MOUNT 0
13690 WHEN "2"
13700 MODE8:
      VDU28,0,31,79,27
13710 COLOUR 131:
      COLOUR 1:
      GCOL 131:
      GCOL 0
13720 CLG:
      PROCsweepaxis
13730 PROCredraw2
13740 PRINT:
      PRINT" Select area to be enlarged using the mouse - Press the LEFT button to exit"
13750 PROCmouse(600,780,120,243,1279,1023)
13760 Xoffset%=xpos%-20:
      Yoffset%=ypos%
13770 TIME=0:
      REPEAT UNTIL TIME>50
13780 CLS
13790 PRINT:
      PRINT" Position comment box using the mouse - Press the LEFT button to exit"
13800 CommentBoxSizeX%=250:
      CommentBoxSizeY%=44
13810 PROCmouse(CommentBoxSizeX%,CommentBoxSizeY%,xpos%,ypos%,600+Xoffset%,780+Yoffset%)
13820 CommentBoxX%=xpos%:
      CommentBoxY%=ypos%
13830 CommentLine1$="Filename: "+FILE1$:
      CommentLine2$="Sweep number "+STR$(a%)+" of "+STR$(m%)+" sweeps"
13840 PROCopenpsfile(-2)
13850 PROCopenpsfile(1)
13860 PROCopenpsfile(2)
13870 OSCLI("ACCESS "+FILE$+" LR")
13880 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
13890 *MOUNT 0
13900 WHEN "3"
13910 MODE8:
      VDU28,0,31,79,27
13920 COLOUR 131:
      COLOUR 1:
      GCOL 131:
      GCOL 0
13930 CLG:
      PROCsweepaxis
13940 PROCredraw2
13950 Ymin=350:
      Xmax=0:
      Ymax=-350
13960 FOR count%=m%(a%-1)+1 TO m%(a%)
13970 IF (D(1,count%)-650)<Ymin THEN Ymin=(D(1,count%)-650):
      Yminpos=count%
13980 IF (D(1,count%)-650)>Ymax THEN Ymax=(D(1,count%)-650):
      Ymaxpos=count%
13990 IF D(0,count%)>Xmax THEN Xmax=D(0,count%):
      Xmaxpos=count%
14000 NEXT
14010 MOVE D(0,Yminpos),D(1,Yminpos):
      DRAW D(0,Yminpos),650
14020 MOVE D(0,Ymaxpos),D(1,Ymaxpos):
      DRAW D(0,Ymaxpos),650
14030 MOVE D(0,Xmaxpos),D(1,Xmaxpos):
      DRAW D(0,Xmaxpos),650
14040 Current_per_mm=NApmm
14050 IF 1000*Current_per_mm>=1000 THEN Current_per_mm=Current_per_mm/1000
14060 IF 1000*Current_per_mm>=1000 THEN Current_per_mm=Current_per_mm/1000
14070 Ipc=ABS((650-D(1,Yminpos))*1250*Current_per_mm/350)
14080 Ipa=ABS((D(1,Ymaxpos)-650)*1250*Current_per_mm/350)
14090 Ispo=ABS((650-D(1,Xmaxpos))*1250*Current_per_mm/350)
14100 IF dr<0 THEN
14110 Analysis_Result=(Ipa/Ipc)+(0.485*Ispo/Ipc)+.086
14120 ELSE
14130 SWAP Ipa, Ipc
14140 Analysis_Result=(Ipc/Ipa)+(0.485*Ispo/Ipa)+.086
14150 ENDIF
14160 CLS
14170 PRINT:
      PRINT" Position comment box using the mouse - Press the LEFT button to exit"
14180 CommentBoxSizeX%=500:
      CommentBoxSizeY%=110
14190 PROCmouse(CommentBoxSizeX%,CommentBoxSizeY%,200,300,1279,1023)
14200 CommentBoxX%=xpos%:
      CommentBoxY%=ypos%
14210 PROCopenpsfile(-3)
14220 PROCopenpsfile(1)
14230 PROCopenpsfile(2)
```

```
14240 OSCLI("ACCESS "+FILE$+" LR")
14250 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
14260 *MOUNT 0
14270 WHEN "4"
14280 MODE8:
      VDU28,0,31,79,27
14290 COLOUR 131:
      COLOUR 1:
      GCOL 131:
      GCOL 0
14300 @%=&0000090A
14310 REM find point of sweep reversal
14320 Xmax=0
14330 FOR count%=m%(a%-1)+1 TO m%(a%)
14340 IF D(0,count%)>Xmax THEN Xmax=D(0,count%):
      Xmaxpos%=count%
14350 NEXT
14360 REPEAT
14370 CLG:
      PROCsweepaxis
14380 PROCredraw2
14390 REPEAT
14400 CLS:
      Diff_step$="":
      PRINT" The number of Data Points for this sweep is ";m%(a%)-m%(a%-1)+1:
      PRINT
14410 INPUT" Enter differentiation step size (1-3)";Diff_step$
14420 UNTIL Diff_step$="1" OR Diff_step$="2" OR Diff_step$="3"
14430 step%=VAL(Diff_step$)
14440 fss%=m%(a%-1)+1
14450 Xmaxpos1%=Xmaxpos%-1-fss%
14460 fse%=fss% + Xmaxpos1% - (Xmaxpos1% MOD step%)
14470 rss%=Xmaxpos%+1
14480 Xmaxpos2%=m%(a%)-rss%
14490 rse%=rss%+Xmaxpos2%-(Xmaxpos2% MOD step%)
14500 Imax=0:
      Imin=0
14510 FOR count%=fss% TO fse%-step% STEP 1
14520 F(0,count%-fss%)=(D(0,count%+step%)+D(0,count%))/2
14530 F(1,count%-fss%)=(D(1,count%+step%)-D(1,count%))/(D(0,count%+step%)-D(0,count%))
14540 IF F(1,count%-fss%) > Imax THEN ImaxF=F(1,count%-fss%)
14550 IF F(1,count%-fss%) < Imin THEN IminF=F(1,count%-fss%)
14560 NEXT
14570 FOR count%=rss% TO rse%-step% STEP 1
14580 R(0,count%-rss%)=(D(0,count%+step%)+D(0,count%))/2
14590 R(1,count%-rss%)=(D(1,count%+step%)-D(1,count%))/(D(0,count%+step%)-D(0,count%))
14600 IF R(1,count%-rss%) > Imax THEN Imax=R(1,count%-rss%)
14610 IF R(1,count%-rss%) < Imin THEN Imin=R(1,count%-rss%)
14620 NEXT
14630 IF Imax < ABS(Imin) THEN Imax=ABS(Imin)
14640 Scale_factor=300/Imax
14650 FOR count%=fss% TO fse%-step% STEP 1
14660 IF count%=fss% THEN
14670 MOVE F(0,count%-fss%),F(1,count%-fss%)*Scale_factor+650
14680 ELSE
14690 DRAW F(0,count%-fss%),F(1,count%-fss%)*Scale_factor+650
14700 ENDIF
14710 NEXT
14720 FOR count%=rss% TO rse%-step% STEP 1
14730 IF count%=rss% THEN
14740 MOVE R(0,count%-rss%),R(1,count%-rss%)*Scale_factor+650
14750 ELSE
14760 DRAW R(0,count%-rss%),R(1,count%-rss%)*Scale_factor+650
14770 ENDIF
14780 NEXT
14790 REPEAT
14800 CLS:
      YN$="":
      PRINT:
      INPUT" Do you want to change differentiation step size Y or N";YN$
14810 UNTIL YN$="Y" OR YN$="N"
14820 UNTIL YN$="N"
14830 REPEAT
14840 CLS:
      YN$="":
      PRINT:
      INPUT" Do you want to enlarge a section of the drawing Y or N";YN$
14850 UNTIL YN$="Y" OR YN$="N"
14860 IF YN$="Y" THEN
14870 PRINT:
      PRINT" Select area to be enlarged using the mouse - Press the LEFT button to exit"
14880 PROCmouse(600,780,120,243,1279,1023)
14890 Xoffset%=xpos%-20:
      Yoffset%=ypos%
14900 TIME=0:
      REPEAT UNTIL TIME>50
14910 CLS
14920 PRINT:
      PRINT" Position comment box using the mouse - Press the LEFT button to exit"
14930 CommentBoxSizeX%=250:
      CommentBoxSizeY%=44
14940 PROCmouse(CommentBoxSizeX%,CommentBoxSizeY%,xpos%,ypos%,600+Xoffset%,780+Yoffset%)
14950 CommentBoxX%=xpos%:
      CommentBoxY%=ypos%
14960 CommentLine1$="Filename: "+FILE1$:
      CommentLine2$="Sweep number "+STR$(a%)+" of "+STR$(m%)+" sweeps"
14970 PROCopenpsfile(-4)
14980 ELSE
14990 PROCopenpsfile(-5)
15000 ENDIF
15010 PROCopenpsfile(1)
15020 PROCopenpsfile(2)
15030 OSCLI("ACCESS "+FILE$+" LR")
15040 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
15050 *MOUNT 0
15060 ENDCASE
15070 UNTIL VAL(analysis_type$)>0 AND VAL(analysis_type$)<6
15080 ENDPROC
15090 :

15100 REM ------------------------------------------------------------------
15110 DEF PROCmouse(boxsizeX%,boxsizeY%,llx%,lly%,urx%,ury%)
```

```
15120 :

15130 REM BOX DRAWING PROCEDURE - BOX IS MOVED USING THE MOUSE
15140 REM boxsizeX%=X dimension of box,boxsizeY%=Y dimension of box
15150 REM llx% and lly% represent the X and Y co-ordinates of the lower left
15160 REM hand corner of the area within which the box is allowed to move.
15170 REM urx% and ury% are co-ordinates of the upper right hand corner of
15180 REM the same area.
15190 :

15200 *POINTER 0
15210 MOVE llx%,lly%
15220 MOUSE ON 0
15230 MOUSE xpos%,ypos%,button%
15240 IF xpos%<llx% THEN xpos%=llx%
15250 IF xpos%>(urx%-boxsizeX%) THEN xpos%=urx%-boxsizeX%
15260 IF ypos%<lly% THEN ypos%=lly%
15270 IF ypos%>(ury%-boxsizeY%) THEN ypos%=ury%-boxsizeY%
15280 MOVE xpos%,ypos%:
      PLOT &06, xpos%+boxsizeX%,ypos%:
      PLOT &06, xpos%+boxsizeX%,ypos%+boxsizeY%:
      PLOT &06, xpos%,ypos%+boxsizeY%:
      PLOT &06, xpos%,ypos%
15290 REPEAT
15300 MOUSE xpos1%,ypos1%,button%
15310 IF xpos1%<llx% THEN xpos1%=llx%
15320 IF xpos1%>(urx%-boxsizeX%) THEN xpos1%=urx%-boxsizeX%
15330 IF ypos1%<lly% THEN ypos1%=lly%
15340 IF ypos1%>(ury%-boxsizeY%) THEN ypos1%=ury%-boxsizeY%
15350 IF xpos1%<>xpos% OR ypos1%<>ypos% THEN
15360 MOVE xpos%,ypos%:
      PLOT &06, xpos%+boxsizeX%,ypos%:
      PLOT &06, xpos%+boxsizeX%,ypos%+boxsizeY%:
      PLOT &06, xpos%,ypos%+boxsizeY%:
      PLOT &06, xpos%,ypos%
15370 MOVE xpos1%,ypos1%:
      PLOT &06, xpos1%+boxsizeX%,ypos1%:
      PLOT &06, xpos1%+boxsizeX%,ypos1%+boxsizeY%:
      PLOT &06, xpos1%,ypos1%+boxsizeY%:
      PLOT &06, xpos1%,ypos1%
15380 xpos%=xpos1%:
      ypos%=ypos1%
15390 ENDIF
15400 UNTIL button%=4
15410 MOUSE OFF:
      button%=0
15420 ENDPROC
15430 :

15440 REM ------------------------------------------------------------------------
15450 DATA "0 0 1280 1010 0","20 0 0 0 0 0",16,1,32,1,3,16,0
15460 DATA 50,"2.54",4,0,1,4,0,0,1,0,1,1,0,"0 0",1,0,",","21 20 0 0 0 0"
```

HPLC Data Collection Program

```
   10 REM >:0.$.HPLCNEW
   20 REM     ============================================================
   30 REM     HPLC DATA COLLECTION PROGRAM - GRJ Harwood - 12/90   v.1.0
   40 REM     ============================================================
   50 :

   60 REM ------------------------------------------------------------------------
   70 REM                         INITIALISATION
   80 CLS:
      CLG:
      MODE8:
      VDU 28,0,31,79,27
   90 *MOUNT 0
  100 COLOUR 131:
      COLOUR 1:
      GCOL 131:
      GCOL 0
  110 CLEAR:
      SET=-1:
      FS$="":
      textwidth=16:
      textheight=16
  120 DIM N(4,100),T(2,100),T$(100),H$(27),S(2,15),D(1,30000)
  130 DIM m%(100),M1%(4,23),T1$(12),T1(4,12),T2(4),CL$(4)
  140 DIM F(1,1000),R(1,1000)
  150 PROCcode
  160 ON ERROR PROCerror
  170 PROCheader
  180 :

  190 REM ------------------------------------------------------------------------
  200 REM                          MAIN MENU
  210 REPEAT
  220 CLS:
      PRINT:
      INPUT " (C)ollect Data, (E)dit or (Q)uit";FS$
  230 CASE FS$ OF
  240 WHEN "C","c"
  250 PROCcollect
  260 WHEN "E","e"
  270 FS$="E"
  280 PROCedit
  290 VDU 28,0,31,79,27
  300 ENDCASE
  310 UNTIL FS$="Q" OR FS$="q"
  320 END
  330 :
```

```
340 REM ---------------------------------------------------------------------
350 DEF PROCcollect
360 B%=0
370 PROCholdinput
380 PROCscale
390 PROCholdaxis
400 CLS:
    PRINT" PRESS ANY KEY TO START":
    IFGET
410 interval=(TS*60/1600)*100
420 TIME=0
430 FINISHTIME=TIME+interval
440 Z$="":
    PROCread
450 IF Z$="E" THEN 500
460 REPEAT
470 CLS:
    YN$="":
    INPUT" Do you want to reset variables  Y or N";YN$
480 UNTIL YN$="Y" OR YN$="N"
490 IF YN$="Y" THEN 370 ELSE 390
500 REPEAT
510 CLS:
    CS$="":
    INPUT" Save (D)ata or (E)xit";CS$
520 CASE CS$ OF
530 WHEN "D","d"
540 CS$="D"
550 PROCsave
560 WHEN "E","e"
570 REPEAT
580 YN$="":
    INPUT" Are you sure Y or N";YN$
590 UNTIL YN$="Y" OR YN$="N"
600 IF YN$="Y" THEN CS$="E" ELSE CS$=""
610 ENDCASE
620 UNTIL CS$="E"
630 ENDPROC
640 :

650 REM ---------------------------------------------------------------------
660 DEF PROCholdinput
670 REPEAT
680 CLS
690 INPUT " TIMESCALE OF data collection in minutes";TS:
    PRINT
700 UNTIL TS>=2.8 AND TS<=72
710 ENDPROC
720 :

730 REM ---------------------------------------------------------------------
740 DEF PROCscale
750 REPEAT
760 CLS:
    PRINT:
    PRINT "                    Please enter AUFS"
770 PRINT" (NB. Detector output should be set to give 1v full scale)";
780 INPUT AUFS
790 UNTIL AUFS>0 AND AUFS<=2
800 step=AUFS/10
810 PRINT
820 SC1$="ABSORBANCE":
    SC2$="AU"
830 ENDPROC
840 :

850 REM ---------------------------------------------------------------------
860 DEF PROCholdaxis
870 CLG
880 VDU5:
    @%=&03
890 MOVE 200,300:
    DRAW1200,300:
    N(1,1)=200:
    N(2,1)=300:
    N(3,1)=1200:
    N(4,1)=300
900 B=0
910 DS=1000/TS
920 IF TS>=20 THEN DS=DS*2
930 IF TS>=40 THEN DS=DS*2
940 IF TS>=60 THEN DS=DS*2
950 FOR A=200 TO 1200 STEP DS
960 MOVEA,295:
    DRAWA,305
970 N(1,B+2)=A
980 N(2,B+2)=295
990 N(3,B+2)=A
1000 N(4,B+2)=305
1010 @%=&01001002
1020 MOVEA-18,282:
     :
     PRINTB:
     T$(B+1)=STR$(B)
1030 T(1,B+1)=A-(textwidth*LEN(T$(B+1))):
     T(2,B+1)=280-textheight
1040 IF TS>=60 THEN JOHN=8
1050 IF (TS>=40 AND TS<60) THEN JOHN=4
1060 IF (TS>=20 AND TS<40) THEN JOHN=2
1070 IF TS<20 THEN JOHN=1
1080 B=B+JOHN
1090 NEXT
1100 B=B+1:
     NT=B
1110 MOVE590,220:
     T$(B)="TIME (minutes)":
     PRINTT$(B):
     T(1,B)=590:
     T(2,B)=220
1120 TT=B
1130 PROCscaledraw2
1140 VDU4
```

```
1150 ENDPROC
1160 :

1170 REM ------------------------------------------------------------------------
1180 DEF PROCread
1190 B%=0:
     C%=0:
     SET=-1
1200 mul%=1
1210 CLS:
     PRINT"Press 'ESC' to halt"
1220 LOCAL ERROR
1230 ON ERROR LOCAL PROCerrorlocal
1240 REPEAT
1250 IF SET <> -1 THEN 1400
1260 IF B%<>0 THEN FINISHTIME=FINISHTIME+interval
1270 VAR1%=1000
1280 store%!12=VAR1%
1290 CALL code%
1300 Y%=store%!4
1310 Y=((Y%/VAR1%)-2048)/2048
1320 D(0,B%)=((TIME/100)*1000/(TS*60)):
     D(1,B%)=(Y*10*700/12)+300+(2*700/12)
1330 PROCdraw
1340 B%=B%+1
1350 IF B%<1023 THEN 1370
1360 *FX 125
1370 REPEAT
1380 UNTIL TIME>FINISHTIME
1390 IF TIME/100 > mul%*TS*60/4 THEN interval=interval*(2^0.5):
     mul%=mul%+1
1400 UNTIL SET <> -1
1410 finish%=B%-1
1420 RESTORE ERROR
1430 ENDPROC
1440 :

1450 REM ------------------------------------------------------------------------
1460 DEF PROCdraw
1470 IF B%=0 THEN
1480 MOVE D(0,B%)+200,D(1,B%)
1490 ELSE
1500 DRAW D(0,B%)+200,D(1,B%)
1510 ENDIF
1520 ENDPROC
1530 :

1540 REM ------------------------------------------------------------------------
1550 DEF PROCscaledraw2
1560 @%=&03
1570 MOVE 200,300:
     DRAW 200,1000
1580 M1%(1,1)=200
1590 M1%(2,1)=300
1600 M1%(3,1)=200
1610 M1%(4,1)=1000
1620 FOR A%=0TO12
1630 B%=(A%*700/12)+300
1640 MOVE 195,B%:
     DRAW 205,B%
1650 M1%(1,A%+2)=195
1660 M1%(2,A%+2)=B%
1670 M1%(3,A%+2)=205
1680 M1%(4,A%+2)=B%
1690 NEXT A%
1700 @%=&0A
1710 @%=EVAL("&01020"+STR$(4-LEN(STR$(AUFS*100)))+"06")
1720 FOR A%=-2 TO 10
1730 B%=(A%*700/12)+300+(700*2/12)
1740 MOVE 80,B%+8
1750 t$=STR$(A%*step):
     NUL$="      ":
     gap$=LEFT$(NUL$,(6-LEN(t$)))
1760 T1$(A%+2)=gap$+t$
1770 PRINT T1$(A%+2):
     T1(1,A%+2)=68:
     T1(2,A%+2)=B%-(textheight/2)
1780 NEXT A%
1790 MOVE 5,662:
     PRINT "AU"
1800 T2(1)=50:
     T2(2)=560:
     T2(3)=50:
     T2(4)=810
1810 @%=&0A
1820 ENDPROC
1830 :

1840 REM ------------------------------------------------------------------------
1850 DEF PROCsure
1860 REPEAT
1870 CLS:
     YN$=""
1880 INPUT"Are you sure Y or N - PREVIOUS GRAPH(S) WILL BE ERASED!";YN$
1890 UNTIL YN$="Y" OR YN$="N"
1900 ENDPROC
1910 :

1920 REM ------------------------------------------------------------------------
1930 DEFPROCerrorlocal
1940 IF ERR<>17 THEN
1950 REPORT:
     PRINT " AT LINE ";ERL;" (Error No. ";ERR;")"
1960 PRINT"PRESS ANY KEY TO CONTINUE"
1970 IFGET
1980 ENDPROC
1990 ENDIF
2000 PROCinterupt
2010 ENDPROC
2020 :

2030 REM ------------------------------------------------------------------------
```

```
2040 DEF PROCinterupt
2050 SET=-1:
     Z$=""
2060 Repeat%=TRUE
2070 REPEAT
2080 CLS:
     PRINT"PRESS 'R' TO RESET - ";
2090 PRINT""
2100 PRINT"PRESS 'E' TO EXIT AND SAVE DATA - PRESS 'X' TO EXIT PROGRAM ";
2110 CASE GET$ OF
2120 WHEN "R","r"
2130 Z$="R":
     Repeat%=FALSE:
     PRINT Z$;:
     INPUT"" SILLY$
2140 WHEN "E","e"
2150 Z$="E":
     Repeat%=FALSE:
     PRINT Z$;:
     INPUT"" SILLY$
2160 WHEN "X","x"
2170 Z$="X":
     Repeat%=FALSE
2180 ENDCASE
2190 UNTIL Repeat%=FALSE
2200 SET=0
2210 IF Z$="X" THEN END
2220 ENDPROC
2230 :

2240 REM ---------------------------------------------------------------------
2250 DEF PROCheader
2260 FOR A%=1 TO 27
2270 READ H$(A%)
2280 NEXT
2290 RESTORE
2300 H$(8)=STR$(textheight)
2310 ENDPROC
2320 :

2330 REM ---------------------------------------------------------------------
2340 DEF PROCsave
2350 REPEAT
2360 CLS:
     FILE1$="":
     INPUT"Data file name ";FILE1$
2370 UNTIL FILE1$<>""
2380 REPEAT
2390 CLS:
     tc$=""
2400 PRINT "          Insert disc (with NAME='Data') in drive 1"
2410 PRINT "               then press C to continue";:
     tc$=GET$
2420 UNTIL tc$="C"
2430 LOCAL ERROR
2440 ON ERROR LOCAL PROCerror
2450 FILE$=FILE1$
2460 *MOUNT 1
2470 OSCLI("SPOOL "+FILE$)
2480 *SPOOL
2490 OSCLI("DELETE "+FILE$)
2500 RESTORE ERROR
2510 CL$(4)="FILENAME: "+FILE$+"    DATE: "+TIME$
2520 channelout= OPENOUT FILE$
2530 PRINT# channelout, FS$
2540 PRINT# channelout, TS
2550 PRINT# channelout, SC1$
2560 PRINT# channelout, step
2570 PRINT# channelout, AUFS
2580 PRINT# channelout, finish%
2590 count%=0:
     flag%=0
2600 REPEAT
2610 PRINT# channelout, D(0,count%)
2620 PRINT# channelout, D(1,count%)
2630 IF D(0,count%)=0 AND D(1,count%)=0 THEN flag%=1
2640 count%=count%+1
2650 UNTIL flag%=1 OR count%=30001
2660 CLOSE# channelout
2670 *MOUNT 0
2680 ENDPROC
2690 :

2700 REM ---------------------------------------------------------------------
2710 DEFPROCerror
2720 CASE ERR OF
2730 WHEN 17
2740 END
2750 WHEN 195
2760 REPEAT
2770 CLS:
     PRINT:
     INPUT"  FILE ALREADY EXISTS - REUSE";YN1$
2780 UNTIL YN1$="Y" OR YN1$="N"
2790 IF YN1$="Y" THEN
2800 OSCLI("ACCESS "+FILE$)
2810 OSCLI("DELETE "+FILE$)
2820 ELSE
2830 REPEAT
2840 FILE1$=FILE$
2850 CLS:
     INPUT"New data file name ";FILE$
2860 UNTIL FILE$<>"" AND FILE$<>FILE1$
2870 FILE1$=""
2880 ENDIF
2890 WHEN 201
2900 CLS:
     PRINT"WRITE PROTECT TAB IS ON - PLEASE REMOVE"
2910 PRINT"PRESS ANY KEY TO CONTINUE":
     IFGET
2920 WHEN 67798
2930 REPEAT
```

```
2940 CLS:
     PRINT"Data file not found - files on disk are:-"
2950 PRINT:
     PRINT"     ";
2960 *INFO ADFS:
     :
     1.$.*
2970 PRINT:
     PRINT"(1) Enter new filename"
2980 PRINT:
     PRINT"(2) Change the disk"
2990 PRINT:
     PRINT:
     INPUT "Please enter your choice: ";selection$
3000 CASE selection$ OF
3010 WHEN "1"
3020 FILE$=FILE1$
3030 REPEAT
3040 PRINT:
     INPUT"New data file name ";FILE1$
3050 UNTIL FILE1$<>"" AND FILE$<>FILE1$
3060 FILE$="":

3070 WHEN "2"
3080 CLS:
     PRINT TAB(10,10);"Press any key when disk has been changed":
     IFGET
3090 *MOUNT 1
3100 ENDCASE
3110 UNTIL selection$="1" OR selection$="2"
3120 OTHERWISE
3130 PRINT
3140 REPORT:
     PRINT " AT LINE";ERL;" (Error No. ";ERR;")";
3150 PRINT" - PRESS ANY KEY TO CONTINUE":
     IFGET
3160 ENDCASE
3170 ENDPROC
3180 :

3190 REM ------------------------------------------------------------------------
3200 DEF PROCcode
3210 osbyte%=6
3220 readbyte%=146
3230 DIM code% 400,store% 20
3240 FOR pass%=0 TO 2 STEP2
3250 P%=code%
3260 [
3270 OPT pass%
3280 STMFD R13!,{R0-R12,R14}
3290 ADR R8,store%
3300 LDR R3,[R8,#12]
3310 MOV R0,#readbyte%
3320 .start
3330 MOV R1,#0
3340 SWI osbyte%
3350 ADDAL R4,R4,R2,LSL#8
3360 MOV R1,#1
3370 SWI osbyte%
3380 ADDAL R4,R4,R2
3390 MOV R1,#2
3400 SWI osbyte%
3410 ADDAL R5,R5,R2,LSL#8
3420 MOV R1,#3
3430 SWI osbyte%
3440 ADDAL R5,R5,R2
3450 MOV R1,#5
3460 SWI osbyte%
3470 ADD R7,R7,#1
3480 TEQ R3,R7
3490 BNE start
3500 MOV R1,#6
3510 SWI osbyte%
3520 ADDAL R6,R6,R2
3530 ADR R8,store%
3540 STMIA R8, {R4,R5,R6}
3550 LDMFD R13!,{R0-R12,PC}
3560 ]
3570 NEXT pass%
3580 ENDPROC
3590 :

3600 REM ------------------------------------------------------------------------
3610 DEFPROCedit
3620 CLS:
     CLG:
     MODE8
3630 COLOUR 131:
     COLOUR 1:
     GCOL 131:
     GCOL 0
3640 REPEAT
3650 CLS:
     FILE1$="":
     INPUT TAB(5,9);"File name of data to be edited";FILE1$
3660 UNTIL FILE1$<>""
3670 REPEAT
3680 CLS:
     tc$=""
3690 PRINT TAB(5,9);"Insert disc in drive 1 then press C to continue";:
     tc$=GET$
3700 UNTIL tc$="C"
3710 LOCAL ERROR
3720 ON ERROR LOCAL PROCerror
3730 *MOUNT 1
3740 PRINT:
     PRINT
3750 OSCLI("INFO "+FILE1$)
3760 PRINT
3770 channelin= OPENIN FILE1$
3780 INPUT# channelin, FS$
3790 INPUT# channelin, TS
```

```
3800 INPUT# channelin, SC1$
3810 INPUT# channelin, step
3820 INPUT# channelin, AUFS
3830 INPUT# channelin, finish%
3840 count%=0
3850 REPEAT
3860 INPUT# channelin, D(0,count%)
3870 INPUT# channelin, D(1,count%)
3880 count%=count%+1
3890 UNTIL EOF# channelin
3900 CLOSE# channelin
3910 OSCLI("ACCESS "+FILE1$+" LR")
3920 RESTORE ERROR
3930 *MOUNT 0
3940 :

3950 REM          OUTPUT FORMAT MENU
3960 REPEAT
3970 CLS
3980 PRINT TAB(10,5);"(1) Produce DESIGNCAD file"
3990 PRINT TAB(10,10);"(2) Produce POSTSCRIPT file"
4000 PRINT TAB(10,15);"(3) Produce ENCAPSUALTED POSTSCRIPT file"
4010 INPUT TAB(15,22);"Please enter your choice: ";output_type$
4020 CASE output_type$ OF
4030 WHEN "1"
4040 PROCoutput_filename
4050 PROCoutput_redraw
4060 PROCdesigncad
4070 WHEN "2"
4080 PROCoutput_filename
4090 PROCoutput_redraw
4100 ps_eps$="PS"
4110 PROCpostscript
4120 WHEN "3"
4130 PROCoutput_filename
4140 PROCoutput_redraw
4150 ps_eps$="EPS"
4160 PROCpostscript
4170 ENDCASE
4180 UNTIL output_type$="1" OR output_type$="2" OR output_type$="3"
4190 ENDPROC
4200 :

4210 REM ------------------------------------------------------------------------
4220 DEF PROCoutput_redraw
4230 VDU 28,0,31,79,27
4240 CLG:
     PROCholdaxis
4250 FOR B%=0 TO finish%
4260 PROCdraw
4270 NEXT B%
4280 ENDPROC
4290 :

4300
4310 REM ------------------------------------------------------------------------
4320 DEF PROCoutput_filename
4330 Disk_Printer$="D"
4340 CASE output_type$ OF
4350 WHEN "1"
4360 output_filename$=FILE1$+"D"
4370 WHEN "2"
4380 output_filename$=FILE1$+"P"
4390 REPEAT
4400 CLS:
     INPUT TAB(10,5);" Output file to (D)isc or (P)rinter";Disk_Printer$
4410 UNTIL Disk_Printer$="D" OR Disk_Printer$="P"
4420 WHEN "3"
4430 output_filename$=FILE1$+"E"
4440 ENDCASE
4450 IF Disk_Printer$="D" THEN
4460 CLS:
     PRINT:
     PRINT
4470 PRINT" Enter NEW OUTPUT FILENAME, or press 'RETURN' to leave unchanged"
4480 PRINT
4490 PRINT"     FILENAME: ";output_filename$;:
     INPUTedit_filename$
4500 IF edit_filename$<>"" THEN output_filename$=edit_filename$
4510 FILE$=output_filename$
4520 ELSE
4530 FILE$="TPIRCSTSOP" :
      REM unlikely filename (POSTSCRIPT backwards)
4540 ENDIF
4550 LOCAL ERROR
4560 ON ERROR LOCAL PROCerror
4570 IF Disk_Printer$="D" THEN
4580 *MOUNT 1
4590 ELSE
4600 *MOUNT 0
4610 ENDIF
4620 OSCLI("SPOOL "+FILE$)
4630 *SPOOL
4640 OSCLI("DELETE "+FILE$)
4650 RESTORE ERROR
4660 ENDPROC
4670 :

4680 REM ------------------------------------------------------------------------
4690 DEF PROCdesigncad
4700 PROCopenfile(-1)
4710 PROCopenfile(0)
4720 OSCLI("ACCESS "+FILE$+" LR")
4730 *MOUNT 0
4740 ENDPROC
4750 :

4760 REM ------------------------------------------------------------------------
4770 DEF PROCpostscript
4780 REPEAT:
     CLS:
     PRINT:
```

```
     INPUT" (P)ortrait or (L)andscape";PL$
4790 UNTIL PL$="P" OR PL$="L"
4800 REPEAT:
     CLS:
     PRINT:
     INPUT" Include the filename on the drawing Y or N";FIL$
4810 UNTIL FIL$="Y" OR FIL$="N"
4820 IF FIL$="Y" THEN PROCcomment_box
4830 IF PL$="P" THEN
4840 PROCopenpsfile(-2)
4850 ELSE
4860 PROCopenpsfile(-1)
4870 ENDIF
4880 PROCopenpsfile(1)
4890 PROCopenpsfile(2)
4900 OSCLI("ACCESS "+FILE$+" LR")
4910 OSCLI("SETTYPE "+FILE$+" POSCRIPT")
4920 *MOUNT 0
4930 IF Disk_Printer$="P" THEN
4940 *MOUNT 0
4950 CLS:
     PRINT:
     PRINT"  Printing - Please Wait......."
4960 OSCLI("ACCESS "+FILE$+" WR")
4970 OSCLI("COPY "+FILE$+" PRINTER: ~C-VDQ")
4980 ENDIF
4990 ENDPROC
5000 :

5010 REM ---------------------------------------------------------------------
5020 DEF PROCopenfile(v%)
5030 LOCAL ERROR
5040 ON ERROR LOCAL PROCerror
5050 IF v%=-1 THEN
5060 OSCLI("SPOOL "+FILE$)
5070 ELSE
5080 OSCLI("SPOOLON "+FILE$)
5090 ENDIF
5100 PROCdatawrite(v%)
5110 *SPOOL
5120 RESTORE ERROR
5130 ENDPROC
5140 :

5150 REM ---------------------------------------------------------------------
5160 DEFPROCdatawrite(w%)
5170 IF w%<>-1 THEN 5520
5180 FOR A%=1TO27
5190 PRINT H$(A%)
5200 NEXT
5210 PRINT "1 2 1 0 0 0":
     PRINT "0 0":
     PRINT "0 0"
5220 PRINT "1 2 1 0 0 0":
     PRINT "0 1010":
     PRINT "0 1010"
5230 PRINT "1 2 1 0 0 0":
     PRINT "1280 1010":
     PRINT "1280 1010"
5240 PRINT "1 2 1 0 0 0":
     PRINT "1280 0":
     PRINT "1280 0"
5250 PRINT "21 0 0 0 0 0"
5260 FOR A%=1 TO NT
5270 PRINT"1 2 1 0 0 1"
5280 PRINT STR$(N(1,A%))+" "+STR$(N(2,A%))
5290 PRINT STR$(N(3,A%))+" "+STR$(N(4,A%))
5300 NEXT A%
5310 @%=&020106
5320 FOR A%=1 TO TT
5330 PRINT"3 1 16 0 0 1"
5340 PRINT STR$(T(1,A%))+" "+STR$(T(2,A%))
5350 PRINT T$(A%)
5360 NEXT
5370 FOR A%=0TO14
5380 PRINT "1 2 1 0 0 1"
5390 PRINT STR$(M1%(1,A%))+" "+STR$(M1%(2,A%))
5400 PRINT STR$(M1%(3,A%))+" "+STR$(M1%(4,A%))
5410 NEXT
5420 FOR A%=0TO12
5430 PRINT "3 1 16 0 0 1"
5440 PRINT STR$(T1(1,A%))+" "+STR$(T1(2,A%))
5450 PRINT T1$(A%)
5460 NEXT
5470 PRINT "3 2 16 0 0 1"
5480 PRINT STR$(T2(1))+" "+STR$(T2(2))
5490 PRINT STR$(T2(3))+" "+STR$(T2(4))
5500 PRINT "SC1"
5510 ENDPROC
5520 PRINT "21 1 0 0 0 0"
5530 f%=finish%:
     IF f%=199 THEN f%=200 ELSE f%=199
5540 FOR b%=0 TO finish% STEP f%
5550 d%=199:
     IF finish%-b%< 200 THEN d%= finish%-b%
5560 @%=&0A
5570 PRINT "1 "+STR$(d%+1)+" 1 0 0 1"
5580 FOR A%=b% TO b%+d%
5590 PRINT STR$(D(0,A%)-D(0,C%)+200)+" "+STR$(D(1,A%))
5600 NEXT
5610 NEXT
5620 ENDPROC
5630 :

5640 REM ---------------------------------------------------------------------
5650 DEF PROCopenpsfile(v%)
5660 LOCAL ERROR
5670 ON ERROR LOCAL PROCerror
5680 IF v%<0 THEN
5690 OSCLI("SPOOL "+FILE$)
5700 ELSE
5710 OSCLI("SPOOLON "+FILE$)
```

```
5720 ENDIF
5730 IF v%<>2 THEN
5740 PROCpswrite(v%)
5750 ELSE
5760 PRINT "state restore"
5770 IF ps_eps$="PS" THEN
5780 PRINT "showpage"
5790 ELSE
5800 PRINT""
5810 ENDIF
5820 ENDIF
5830 *SPOOL
5840 RESTORE ERROR
5850 ENDPROC
5860 :

5870 REM ------------------------------------------------------------------------
5880 DEF PROCpswrite(w%)
5890 REM  - Postscript file writing routine -
5900 REM  - if w%<0 = Write Header as follows
5910 REM  -     w%=-1 = Standadrd sized landscape drawing
5920 REM  -     w%=-2 = Standadrd sized portrait drawing
5930 REM  - if w%>0 = Write Curve Data
5940 REM
5950 @%=&01020206
5960 IF w%>0 THEN 7090
5970 IF ps_eps$="EPS" THEN
5980 PRINT"%!PS-Adobe-2.0 EPSF-1.2"
5990 PRINT"%%Creator: HPLC data collection program"
6000 PRINT"%%For: WordPerfect input"
6010 PRINT"%%Title: ";FILE$
6020 PRINT"%%CreationDate: ";TIME$
6030 IF w%=-1 THEN
6040 PRINT"%%BoundingBox: 0 0 712 792"
6050 ELSE
6060 PRINT"%%BoundingBox: 0 0 576 405"
6070 ENDIF
6080 PRINT"%%DocumentFonts: Times-Roman"
6090 PRINT"%%EndComments"
6100 ELSE
6110 PRINT "%!";
     PRINT "%%EndComments"
6120 ENDIF
6130 PRINT
6140 PRINT "%----------------Prologue---------------"
6150 PRINT "/_rm"
6160 PRINT "     {rmoveto} bind def"
6170 PRINT "/_rl"
6180 PRINT "     {rlineto} bind def"
6190 PRINT "/_s"
6200 PRINT "     {show} bind def"
6210 PRINT
6220 PRINT "%%EndProlog"
6230 PRINT
6240 PRINT "%-----------------SCRIPT----------------"
6250 PRINT "/state save def"
6260 PRINT
6270 PRINT "%---------------Define Fonts------------"
6280 PRINT "%large font lf"
6290 PRINT "/_lf"
6300 PRINT "     /Times-Roman findfont 24 scalefont def"
6310 PRINT
6320 PRINT "%medium font mf"
6330 PRINT "/_mf"
6340 PRINT "     /Times-Roman findfont 20 scalefont def"
6350 PRINT
6360 PRINT "%small font sf"
6370 PRINT "/_sf"
6380 PRINT "     /Times-Roman findfont 16 scalefont def"
6390 PRINT
6400 PRINT "%---------Move Origin and Scale---------"
6410 IF w%=-1 THEN
6420 PRINT "%Landscape orientation"
6430 PRINT "20 792 translate"
6440 PRINT "-90 rotate"
6450 PRINT ".566929 dup scale"
6460 ELSE
6470 PRINT "%Portrait orientation"
6480 PRINT ".45 dup scale"
6490 ENDIF
6500 PRINT
6510 PRINT "%----Translation before drawing Axis----"
6520 IF w%=-1 THEN
6530 PRINT "0 -40 translate"
6540 ELSE
6550 PRINT "0 -180 translate"
6560 ENDIF
6570 PRINT
6580 IF FIL$="Y" THEN
6590 PRINT "%-----------Draw Comment Box------------"
6600 PRINT STR$(CommentBoxX%)+" "+STR$(CommentBoxY%)+" moveto"
6610 PRINT "%store box position in postscript variables Xpos and Ypos"
6620 PRINT "currentpoint"
6630 PRINT "/Ypos exch def"
6640 PRINT "/Xpos exch def"
6650 PRINT STR$(CommentBoxSizeX%)+" 0 _rl"
6660 PRINT "0 "+STR$(CommentBoxSizeY%)+" _rl"
6670 PRINT "-"+STR$(CommentBoxSizeX%)+" 0 _rl"
6680 PRINT "closepath"
6690 PRINT
6700 PRINT "%---------Fill Comment Box -------------"
6710 PRINT "_sf setfont"
6720 PRINT "Xpos Ypos moveto"
6730 PRINT "6 22 _rm"
6740 PRINT "("+CommentLine1$+") _s"
6750 PRINT "Xpos Ypos moveto"
6760 PRINT "6 6 _rm"
6770 PRINT "("+CommentLine2$+") _s"
6780 PRINT
6790 ENDIF
6800 PRINT "%---------------Draw Axis---------------"
6810 PRINT "_lf setfont"
```

```
6820 FOR A%=1 TO NT
6830 PRINT STR$(N(1,A%))+" "+STR$(N(2,A%))+" moveto"
6840 PRINT STR$(N(3,A%))+" "+STR$(N(4,A%))+" lineto"
6850 PRINT "stroke"
6860 NEXT A%
6870 FOR A%=1 TO TT
6880 PRINT STR$(T(1,A%)+16)+" "+STR$(T(2,A%))+" moveto"
6890 PRINT "("+T$(A%)+") _s"
6900 NEXT
6910 FOR A%=1TO14
6920 PRINT STR$(M1%(1,A%))+" "+STR$(M1%(2,A%))+" moveto"
6930 PRINT STR$(M1%(3,A%))+" "+STR$(M1%(4,A%))+" lineto"
6940 PRINT "stroke"
6950 NEXT
6960 FOR A%=0TO12
6970 PRINT STR$(T1(1,A%)+62)+" "+STR$(T1(2,A%))+" moveto"
6980 PRINT "("+T1$(A%)+") _s"
6990 NEXT
7000 PRINT STR$(T2(1)+50)+" "+STR$(T2(2))+" translate"
7010 PRINT "90 rotate"
7020 PRINT "0 0 moveto"
7030 PRINT "("+SC1$+") _s"
7040 PRINT "-90 rotate"
7050 PRINT "-"+STR$(T2(1)+50)+" -"+STR$(T2(2))+" translate"
7060 PRINT "0.5 setlinewidth"
7070 PRINT
7080 ENDPROC
7090 FOR A%=0 TO finish%
7100 IF A%=0 THEN
7110 PRINT STR$(D(0,A%)+200)+" "+STR$(D(1,A%))+" moveto"
7120 ELSE
7130 PRINT STR$(D(0,A%)+200)+" "+STR$(D(1,A%))+" lineto"
7140 ENDIF
7150 NEXT
7160 PRINT "stroke"
7170 ENDPROC
7180 :

7190 REM ------------------------------------------------------------------------
7200 DEF PROCcomment_box
7210 CLS
7220 PRINT:
     PRINT" Position comment box using the mouse - Press the LEFT button to exit"
7230 CommentBoxSizeX%=250:
     CommentBoxSizeY%=44
7240 PROCmouse(CommentBoxSizeX%,CommentBoxSizeY%,200,300,1279,1023)
7250 CommentBoxX%=xpos%:
     CommentBoxY%=ypos%
7260 CommentLine1$="Filename: "+FILE1$
7270 REPEAT
7280 CLS:
     PRINT:
     INPUT" Please enter comment";CommentLine2$
7290 PRINT:
     INPUT " OK - Y or N";YN$
7300 UNTIL YN$="Y" OR YN$="y"
7310 ENDPROC
7320 :

7330 REM ------------------------------------------------------------------------
7340 DEF PROCmouse(boxsizeX%,boxsizeY%,llx%,lly%,urx%,ury%)
7350 :

7360 REM BOX DRAWING PROCEEDURE - BOX IS MOVED USING THE MOUSE
7370 REM boxsizeX%=X dimension of box,boxsizeY%=Y dimension of box
7380 REM llx% and lly% represent the X and Y co-ordinates of the lower left
7390 REM hand corner of the area within which the box is allowed to move.
7400 REM urx% and ury% are co-ordinates of the upper right hand corner of
7410 REM the same area.
7420 :

7430 *POINTER 0
7440 MOVE llx%,lly%
7450 MOUSE ON 0
7460 MOUSE xpos%,ypos%,button%
7470 IF xpos%<llx% THEN xpos%=llx%
7480 IF xpos%>(urx%-boxsizeX%) THEN xpos%=urx%-boxsizeX%
7490 IF ypos%<lly% THEN ypos%=lly%
7500 IF ypos%>(ury%-boxsizeY%) THEN ypos%=ury%-boxsizeY%
7510 MOVE xpos%,ypos%:
     PLOT &06, xpos%+boxsizeX%,ypos%:
     PLOT &06, xpos%+boxsizeX%,ypos%+boxsizeY%:
     PLOT &06, xpos%,ypos%+boxsizeY%:
     PLOT &06, xpos%,ypos%
7520 REPEAT
7530 MOUSE xpos1%,ypos1%,button%
7540 IF xpos1%<llx% THEN xpos1%=llx%
7550 IF xpos1%>(urx%-boxsizeX%) THEN xpos1%=urx%-boxsizeX%
7560 IF ypos1%<lly% THEN ypos1%=lly%
7570 IF ypos1%>(ury%-boxsizeY%) THEN ypos1%=ury%-boxsizeY%
7580 IF xpos1%<>xpos% OR ypos1%<>ypos% THEN
7590 MOVE xpos%,ypos%:
     PLOT &06, xpos%+boxsizeX%,ypos%:
     PLOT &06, xpos%+boxsizeX%,ypos%+boxsizeY%:
     PLOT &06, xpos%,ypos%+boxsizeY%:
     PLOT &06, xpos%,ypos%
7600 MOVE xpos1%,ypos1%:
     PLOT &06, xpos1%+boxsizeX%,ypos1%:
     PLOT &06, xpos1%+boxsizeX%,ypos1%+boxsizeY%:
     PLOT &06, xpos1%,ypos1%+boxsizeY%:
     PLOT &06, xpos1%,ypos1%
7610 xpos%=xpos1%:
     ypos%=ypos1%
7620 ENDIF
7630 UNTIL button%=4
7640 MOUSE OFF:
     button%=0
7650 ENDPROC
7660 :

7670 REM ------------------------------------------------------------------------
7680 DATA "0 0 1280 1010 0","20 0 0 0 0 0",16,1,32,1,3,16,0
```

```
7690 DATA 50,"2.54",4,0,1,4,0,0,1,0,1,1,0,"0 0",1,0,*,"21 20 0 0 0 0"
```